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VOL. 27, SEC. E. JUNE, 1949 NUMBER 3

THE DETERMINATION OF PHOSPHOLIPID PHOSPHORUS[1]

BY J. M. R. BEVERIDGE AND S. E. JOHNSON

Abstract

A simple accurate method for the determination of phospholipid phosphorus is described. Conditions for the digestion of phospholipid have been developed permitting the utilization of a colorimetric procedure for inorganic phosphorus that is characterized by excellent color stability and strict adherence to Beer's law over a range of 0 to 65 μgm. of phosphorus.

Introduction

The existence of such a large number of modifications for the determination of phosphorus by the "molybdenum blue" procedure (Woods and Mellon (5)) attests to the fact that these methods have not been entirely satisfactory.

Following a trial of one of these procedures (King (3)) with results that were undesirable (cf. also Allen (1)), a review of the literature was made to find a suitable method or one that could be adapted readily to the requirements of the work to be performed. These were:

1. The rapid digestion of organic matter without loss of phosphorus.

2. A stability of color that would permit the comparison of a large number of samples at one time.

3. Adherence to Beer's law of the relationship between phosphorus concentration and color density over a range of 5 to 65 μgm. of phosphorus.

4. A degree of error not more than approximately 1% in the colorimetric procedure.

Experimental

After a survey of a number of the modifications of the molybdenum blue method, it was decided that the procedure for inorganic phosphorus described by Boltz and Mellon (2) appeared to hold most promise for adaptation to the determination of phospholipid phosphorus. These workers showed that the blue color obtained when molybdic acid itself is reduced differs from that obtained from the reduction of the phosphoric – molybdic acid complex. The former absorbs light most strongly in the region of 630 to 650 mμ, whereas the latter color, termed heteropoly blue to distinguish it from molybdenum blue, has a characteristic absorption band at 820 to 830 mμ.

[1] *Manuscript received February 3, 1949.*

A contribution from the Department of Pathological Chemistry, Faculty of Medicine, University of Western Ontario, London, Ont., with financial assistance from the Life Insurance Medical Research Fund, New York, U.S.A.

They stated that the color was stable for 12 hr. and that Beer's law held over the range 0 to 1.5 parts per million (0 to 75 μgm.) of phosphorus. An error of 2% was considered to be negligible.

These statements were confirmed and, since they were not accompanied by illustrative data in the original paper, the results of the present work are given here. Fig. 1 shows the relationship between color density and phosphorus

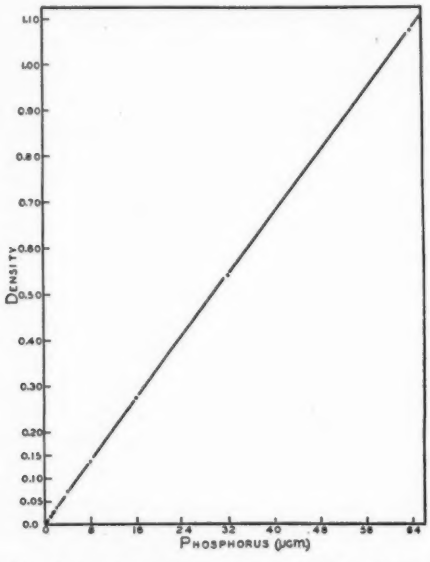

FIG. 1. *Relationship of phosphorus concentration to density of "heteropoly" blue color.*

concentration. Table I reveals that there is excellent color stability up to at least 23 hr., beyond which time it was not tested. An examination of the data in the table also shows that the percentage error is of the order of only 1% or less.

TABLE I

STABILITY OF HETEROPOLY BLUE COLOR

Sample	Density	
	1 Hr.	23 Hr.
1	0.338	0.339
2	0.343	0.341
3	0.338	0.337
4	0.342	0.340
5	0.336	0.335
6	0.335	0.333
7	0.337	0.335
8	0.342	0.339

The problem, therefore, resolved itself into the elaboration of adequate conditions for the digestion of the phospholipid that would permit the use of this excellent colorimetric procedure. Since the color development took place in 1*N* sulphuric acid, it was obvious that the digestion had to be carried out in this medium. In the digestion of organic matter for the determination of phosphorus, the following points must be taken into consideration.

1. The digestion should be rapid and complete.

2. There must be no significant loss of sulphuric acid since this would affect markedly subsequent color development by permitting the reduction of molybdic acid itself with the formation of molybdenum blue.

3. There must be no loss of phosphoric acid. The possibility of error being introduced by this factor is very real since phosphoric acid is volatile at a temperature of 213° C., whereas concentrated sulphuric acid boils at 330° C.

4. There must be no conversion of orthophosphoric acid to meta- and pyrophosphoric acids or, if there is any such conversion, it must be reversed since the latter compounds do not react to give a blue color.

Various combinations of potassium sulphate, copper sulphate, selenium dioxide, and mercuric oxide were used as catalysts to speed the oxidation of the total organic matter by heating with 1 ml. of concentrated sulphuric acid. The digestion time was reduced to between 5 and 10 min. but all these catalysts were found to interfere to some degree with subsequent color development. Consequently, attempts to utilize them were abandoned.

Finally, the following procedure was adopted. The phospholipids were precipitated according to the method of Sinclair and Dolan (4) in a Pyrex test tube about 15 cm. in length, 1.4 cm. in diameter, and about 20 ml. in capacity. Any organic solvent remaining after the tubes were drained was completely driven off. One ml. of concentrated sulphuric acid was added and the tube heated over the low flame of a microburner for about five minutes. Two drops of 30% hydrogen peroxide were then added to the charred mixture, shaking thoroughly between each drop. This treatment practically completed the oxidation. Heating was continued a further 10 min. to ensure completion of oxidation and destruction of excess hydrogen peroxide. If any of the latter substance is allowed to persist, color development is seriously inhibited.

In order to determine the minimal length of time necessary for complete combustion and to ascertain whether or not any significant loss of sulphuric and phosphoric acids occurred during digestion, the following experiment was performed. Samples of lipid extract were digested in the usual way for periods up to 60 min. Glass funnels with stems drawn to a fine tip and sealed with a drop of water were placed in a number of the digestion tubes to prevent loss of sulphuric and phosphoric acids. The results are shown in Table II and indicate that a 15 min. digestion time is adequate. Since there is no difference in the results derived from the series with and without funnels, it may be concluded that no significant loss of either sulphuric or phosphoric

acid occurs under the conditions employed. Titrimetric data for the former acid support this conclusion.

TABLE II

CONDITIONS FOR DIGESTION OF PHOSPHOLIPID

	Time	
	15 min.	60 min.
	Density	
With funnel	0.338	0.336
Without funnel	0.342	0.339
Diluted digest boiled	0.342	

The possibility that the conditions of the combustion effected a conversion of orthophosphoric acid to the meta- and pyro-forms was investigated by boiling the digested sample with 24 ml. of water for about 10 min. This treatment reconverts any of the latter compounds to orthophosphoric acid. The data obtained are shown also in Table II. If meta- and pyrophosphoric acids had been present, the unboiled samples would have given lower results since these forms do not react with molybdic acid to yield a compound that, on reduction, will form a blue color. The close similarity between the amounts of phosphorus found in the boiled and unboiled samples precludes the existence of any such conversion under these conditions.

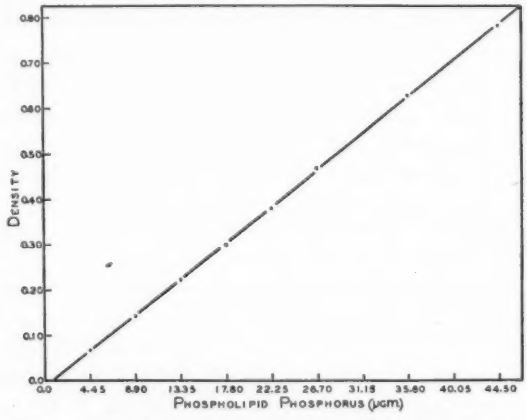

FIG. 2. *Effect of sample size on phospholipid determination.*

In view of the marked sensitivity of the colorimetric procedure, the question arose regarding the minimal aliquot of mixed lipids that could be used and that would still permit a reasonable degree of accuracy in the determination of phospholipids. This point was investigated by the precipitation of the

phospholipids in varying aliquots of a crude lipid extract according to the method of Sinclair and Dolan (4). The precipitates were digested as previously described and total phosphorus determined. The results, in Fig. 2, reveal that amounts of phospholipid as low as 0.1 mgm., equivalent to approximately 4 μgm., may be utilized without sacrifice of accuracy.

The method in detail is given herewith.

1. Add 1* ml. of concentrated sulphuric acid to the phospholipid sample in a Pyrex test tube.

2. Heat for five minutes over the low flame of a microburner; add two drops of 30% hydrogen peroxide (low in phosphorus), shaking between the addition of the drops. Heat 10 min. longer.

3. Cool; add 9* ml. of distilled water and transfer to a 50 ml. volumetric flask with the aid of 15* ml. of distilled water.

4. Add 20* ml. of freshly prepared molybdate – hydrazine sulphate reagent** from a rapid delivery pipette.

5. Make the solution up to volume with distilled water, mix, loosen stopper, and place the flask in a boiling water bath for 10 min.

6. Remove and cool the flask rapidly by placing it in a cold water bath until the level of the liquid again reaches the mark.

7. Measure the color density within 24 hr. at 830 mμ.

Blank determinations were performed on the reagents using 1 ml. of concentrated sulphuric acid and two drops of 30% hydrogen peroxide. The amount of color obtained was equivalent to only 0.26 μgm. of phosphorus. It may be noted that this amount represents about 1% at a level of 20 μgm. of phosphorus in the test sample.

This method affords an accurate, rapid determination of phospholipid phosphorus and, because of the color stability, it lends itself admirably to the performance of a large number of analyses at a time.

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** These volumes are not critical.*

*** This reagent was originally described by Boltz and Mellon as being prepared by combining 25 ml. 2.5% (W/V) sodium molybdate dihydrate in 10N sulphuric acid with 10 ml. of 0.15% (W/V) hydrazine sulphate and making up to 100 ml. with distilled water. The amount of acid contained in 20 ml. of this reagent made the final solution 1N with respect to sulphuric acid.*

Since the concentrated sulphuric acid used in the digestion process contained about 35 m. e., the sodium molybdate had to be dissolved in more dilute acid. A concentration of 3N sulphuric acid yielded the necessary m. e. to make the final solution 1N with respect to acid.

AGGLUTINATION OF ERYTHROCYTES CONTAINING SULPHAEMOGLOBIN – FURTHER EVIDENCE OF SPECIFIC AGGREGATION[1]

By Kenneth W. McKerns and Orville F. Denstedt

Abstract

By sulphuration of red blood cells with hydrogen sulphide, up to 75% of the haemoglobin can be converted to sulphaemoglobin without seriously impairing the osmotic stability or the agglutinability of the cells with various antisera with exception of anti-Rh. Unlike the A, B, M, and N agglutinogens, the Rh factor is labile to sulphuration and numerous other treatments. Sulphurated cells, by their size and color, can be distinguished from normal cells under the microscope. This feature has been used to confirm Marrack's hypothesis that the aggregation phase of the agglutination reaction is specific.

The agglutination of erythrocytes, bacteria, and colloidal antigens by specific antisera is commonly regarded as being a two stage process. In the first stage, antibody of the serum combines with antigen on the cells or particles, producing a state of "sensitization." In the second, or "aggregation" stage, the cells or particles cohere and precipitate out in clumps. Sensitization is highly specific, but whether the aggregation process also is specific is more difficult to prove. Bordet (3) maintained that the latter is nonspecific; that combination of antigen with antibody so alters the properties of the cells or particles as to render them susceptible to flocculation by the electrolyte in the surrounding medium.

Several years later Marrack (16) advanced the theory of specific aggregation, frequently referred to as the "framework" or "lattice" hypothesis, which implies that homologous antibodies constitute the links that hold aggregated cells or particles together. Thus, if cells, differing in antigen composition, be mixed and agglutinated simultaneously with their respective homologous antisera, the aggregates according to Bordet, should consist of a mixture, whereas according to Marrack each type of cell should form separate aggregates.

The preponderance of experimental evidence supports Marrack's hypothesis. Heidelberger and Kendall (8-12) precipitated polysaccharide, azoprotein, and other protein antigens with immune serum, and showed that aggregation is specific. Topley, Wilson, and Duncan (27) used a mixture of two species of bacteria to demonstrate that simultaneous agglutination with the respective homologous antisera caused the organisms to form separate clumps. Wiener and Herman (29) also obtained discrete clumps with a mixture of Kline antigen and human red corpuscles. The classic studies of Pauling and his associates (20-25) with coupled azoprotein compounds provide especially strong evidence in support of Marrack's hypothesis. On the other hand, Abramson (1) obtained both homogeneous and heterogeneous clumps with a mixture of sheep

[1] *Manuscript received February 1, 1949.*
Contribution from the Department of Biochemistry, McGill University, Montreal, Que.

red cells and Friedlander bacilli. Likewise, Hooker and Boyd (13) mixed mammalian and avian red corpuscles with the respective antisera and obtained heterogeneous clumps suggestive of nonspecific aggregation. Later, however, Abramson and Boyd (2) repeated the experiments with partially haemolyzed red cells and observed a tendency towards specific aggregation.

Duncan (5), in some measure reconciles the two hypotheses. On agglutinating mixtures of flagellated and nonflagellated bacteria with immune sera, he obtained conclusive evidence that both stages of agglutination were specific. In precipitin test with mixtures of yeast polysaccharide and crystalline albumin, however, there was evidence of both modes of aggregation, the primary mechanism being specific and followed by a secondary nonspecific aggregation.

The kinetics of agglutination based on the Bordet hypothesis has been studied by Teorell (26) and by Morales (17), who have derived equations on the assumption of a univalent type of antibody and a polyvalent antigen. Analogous studies based on the specific aggregation hypothesis have been reported by Heidelberger and others (7). The mathematical treatments have been reviewed by Boyd (4), while the fundamental mechanism of the reaction and the nature of the forces involved have been discussed by Pauling (18, 19).

Further confirmation of Marrack's hypothesis has now been obtained in our laboratory by use of a mixture of normal and sulphurated erythrocytes. As shown below, red cells can be sulphurated *in vitro* to a high content of sulphaemoglobin without altering their agglutinability. The sulphurated cells are pale green and are slightly larger in diameter than normal cells. On agglutination they undergo less crenation and shrinkage in diameter than normal cells, and thus are readily distinguishable from the latter in an agglutinated mixture. The experiments here reported concern the influence of sulphuration on agglutinability of human red corpuscles and use of sulphurated cells in confirming the specific aggregation hypothesis.

Experimental

Preparation of Sulphurated Red Cells

A suspension (about 5%) of human red cells from a few drops of blood was made with isotonic phosphate buffer* of pH 7.35 in a small serological test tube (10 × 75 mm.). The air in the tube was displaced with hydrogen sulphide, and the tube was stoppered and slowly and repeatedly inverted. Addition of hydrogen sulphide and mixing were repeated two or three times depending on the degree of sulphuration desired. After standing for 20 min. at room temperature with occasional mixing, the red cells assumed a deep greenish color. To remove residual hydrogen sulphide the tube was centrifuged lightly, the supernatant fluid removed by aspiration, and the cells

** The buffer mixture was prepared by mixing 20 ml. of 4.17% sodium dihydrogen phosphate monohydrate and 16.5 ml. of 0.302 N sodium hydroxide.*

washed with the phosphate buffer solution. Washing was repeated until the supernatant gave a negative test with lead acetate. The proportion of sulphaemoglobin to haemoglobin in the cells was determined by the method of Evelyn and Malloy (6). No attempt was made to distinguish between sulphaemoglobin and choleglobin (15), which, if present, would be measured as sulphaemoglobin. To ensure ready differentiation of the sulphurated and normal cells in agglutination experiments, the sulphaemoglobin content of the cells should be between 50 and 75% of the total pigment.

Stability and Agglutinability of Sulphurated Red Cells

It is important in agglutination studies that the stability and agglutinability of the cells should not be greatly altered by sulphuration. The following tests were performed to ascertain the effect of the treatment on these properties.

Stability

One ml. of lightly packed washed cells was suspended in 2 ml. of isotonic phosphate buffer (pH 7.35); 0.5 ml. portions of the suspension were dispensed into serological tubes. Hydrogen sulphide was introduced into the space above the liquid and the tubes were stoppered and agitated gently for 20 min. The excess hydrogen sulphide was then removed with the aid of an aspirating tube.

Osmotic stability was tested by the fragility method of Waugh and Asherman (28), who recommend the use of a buffered saline (Simmel's) in place of simple sodium chloride solutions.

The sulphurated cell suspension was pipetted in 0.02 ml. portions into 15 ml. centrifuge tubes containing isotonic Simmel's solution and a range of hypotonic concentrations. After five minutes the tubes were centrifuged for five minutes. The supernatant solution was discarded and the cell sediment suspended in 10 ml. of $M/60$ phosphate buffer of pH 6.6. One drop of each of the following solutions: concentrated ammonium hydroxide, 20% potassium ferricyanide, and 10% potassium cyanide was added in that order. The concentration of cyanmethaemoglobin (cyanhaemochromogen) was determined by Evelyn's method (6), with a 540 mμ filter.

TABLE I

CELL HAEMOLYSIS AFTER FIVE MINUTES IN SIMMEL'S SOLUTION

Pigment as SHb, %	Dilution, % of isotonic				
	100	70	60	50	40
	Haemolysis, %				
0	0	0	0	0	81
35	0	0	0	0	84
45	0	0	0	11	85
75	0	0	10	54	95

It will be observed from Table I that the osmotic fragility of the cells to hypotonic saline solutions is not greatly increased by sulphuration *in vitro*. For the present purpose the slightly increased fragility is of no consequence, since cells containing from 50 to 60% of sulphaemoglobin are stable for several hours in saline even down to 60% of the isotonic concentration. As all the agglutination tests were carried out in isotonic medium no breakdown of the sulphurated cells occurred during the 15 min. period of the test.

Agglutinability

Two criteria were adopted to validate the use of sulphaemoglobin for labelling the cells: (1) comparison of sulphurated and normal cells in titer tests with a given potent antiserum and (2) comparison of the number of free cells after agglutination of the sulphurated and normal cells.

(1) Titer Tests

When the potency of various strong antisera, including anti-A, anti-B, anti-M, anti-N, anti-Rh_o (anti-D), and anti-Rh″ (anti-E), was tested with appropriate cells, both normal and sulphurated cells from a given donor were found to give the same titer values except when anti-Rh sera were used. The Rh agglutinogens were found to be labile to numerous types of treatment whereas the A, B, M, and N antigens are very stable. Thus a lower titer was obtained with the Rh antisera especially when the sulphaemoglobin level in the cells was more than 50%. Obviously there is no change in the potency of the serum but the impaired reactivity of the Rh antigen results in a lower titer value.

(2) Free-cell Counts

Fresh blood was drawn up to the 1.0 mark in a white-cell pipette, which then was filled to the 11 mark with isotonic phosphate buffer of pH 7.4. After the pipette was shaken, the contents were transferred to a serological tube and an equal amount of buffer was added. The contents were sulphurated and gently agitated mechanically for 20 min. Finally, excess hydrogen sulphide was removed. Colloidal sulphur frequently is formed during sulphuration but is of no consequence.

Duplicate cell suspensions were prepared from blood from various Group A individuals according to the procedure outlined. One sample from each pair was sulphurated and then all samples were agglutinated with potent anti-A serum and the unagglutinated cells were counted according to the following procedure.

Blood is drawn up to the 1.0 mark in a white-cell pipette, which then is filled to the 11 mark with agglutinating serum. The pipette is shaken and the contents transferred to a serological tube. The tubes are gently agitated four or five times during a two minute period and then centrifuged for exactly two minutes at 1500 r.p.m. After flicking the tube to dislodge the agglutinated cell sediment the sample is agitated mechanically for 40 min. at the rate of 230 vibrations per minute.

For counting the free cells, the larger clumps are permitted to settle for about three seconds and a sample of the supernatant fluid is transferred to the haemocytometer by means of a white-cell pipette filled to the 1.0 mark. The free cells in the entire field (144 large squares) are counted and the number of cells per cu. mm. calculated as follows:

$$\text{Free cells per cu. mm.} = \frac{\text{total count} \times 11 \times 22 \times 10}{9}$$

$$= \text{total cell count by } 269 .$$

Results:

(*a*) Reproducibility of free-cell count.

The following figures were obtained from four successive agglutination tests on Group A red cells containing 71% sulphaemoglobin: 19,900; 18,300; 20,200; 20,100. Mean 19,625.

(*b*) Comparison of free-cell counts on normal and sulphurated cells.

The cell counts given in Table II represent the means of duplicate counts. The cells were taken from different donors, all of group ARh_o. In all instances anti-A serum of 512 titer was used.

TABLE II

FREE-CELL COUNT BEFORE AND AFTER SULPHURATION

Subject	Cell specimens	SHb, %	Duration of agitation at 230 vibrations per min.	Free-cells per cu. mm.
I	Normal	0	55	14,200
	Sulphurated	60	55	15,000
II	Normal	0	40	21,000
	Sulphurated	75	40	22,600
III	Normal	0	30	39,900
	Sulphurated	67	30	38,000
IV	Normal	0	45	53,000
	Sulphurated	70	45	51,400

It is evident that the reproducibility of the counts is satisfactory and that the agglutinability of the cells is not altered by the sulphuration treatment.

Use of Sulphurated Red Cells for Testing Marrack's Hypothesis

Sulphurated red cells, which are thus shown to be comparable in stability and agglutinability with normal cells, can be used for testing the specificity of aggregation. The general procedure was as follows: light saline suspensions of normal and sulphurated human erythrocytes were adjusted by dilution so that the concentration of cells in all tubes was the same. To facilitate differentiation of the normal and sulphurated cells in the aggregates, the serum was diluted so as to favor the production of small rather than large clumps. One

PLATE I

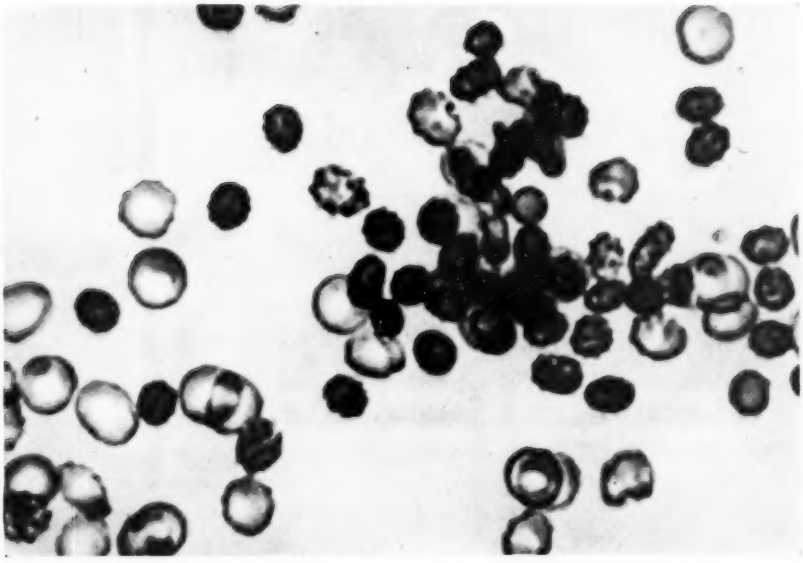

FIG. 1. *Appearance of normal and sulphurated red corpuscles in an agglutinated mixture.*

drop of each of the two cell suspensions was introduced into a serological tube with two drops of the appropriate antiserum or sera. The tube was centrifuged at low speed (about 1500 r.p.m.) for two minutes. The cell sediment was then broken into small clumps by flicking the tube. The microphotograph in Fig. 1 illustrates the difference in appearance of the normal and sulphurated erythrocytes. If a daylight lamp is used, the sulphurated cells appear larger, paler, and slightly greenish in color. Table III gives the results of a typical experiment.

TABLE III

SPECIFIC AGGLUTINATION WITH A AND B AGGLUTINOGENS

Blood group of red cells in mixture	Agglutinating serum	Composition of resulting clumps
A + A	Anti-A (titer 512)	Mixed *AA*
A + B	Anti-B (titer 512)	B only
A + B	Anti-A	A only
A + B	Anti-A + Anti-B	Separate *A* and B

Letters in italics designate sulphurated cells.

Analogous results were obtained with cell mixtures *B* + B, *B* + A, *M* + M, *M* + N, M + *N*, *Rh*$_o$ + Rh$_o$, and *Rh*$_o$ + Rh″ with the appropriate sera.

For the M- and N-tests, red cells of group BMRh$_o$ (50% SHb) and group ANRh$_o$ (46% SHb) were used with anti-M and anti-N sera (titer 32). The anti-M serum was absorbed with a light suspension of "N" cells, and the anti-N, with "M" cells, prior to using.

In the case of the Rh antigens the Rh$_o$ cells were sulphurated to a level of 45% SHb. Anti-Rh$_o$ (anti-D) and anti-Rh″ (anti-E) sera of titer 64 were used.

Discussion and Conclusions

From the foregoing results it is clear that sulphuration *in vitro* with hydrogen sulphide does not seriously alter the osmotic stability of human red cells, nor does it affect the agglutinability except with anti-Rh serum. The Rh antigen, being much more labile to various treatments than the A, B, M, or N antigens, is altered by sulphuration, especially when the sulphaemoglobin is about 50% of the total pigment. The A, B, M, and N antigens, on the other hand, are stable up to 75% sulphuration, which is the maximum level attainable by our method.

Examination of the clumps obtained by agglutinating mixtures of normal and sulphurated red cells of various types with appropriate antisera showed consistently that cells of different types formed discrete aggregates. In other words, both sensitizing and aggregating phases of the agglutination reaction appear to be specific, as postulated by Marrack and others.

Acknowledgments

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THE EFFECT OF ANTITOXIN ON EXPERIMENTAL INFECTION WITH *CLOSTRIDIUM NOVYI* (*OEDEMATIENS*) IN GUINEA PIGS, WITH A NOTE ON ACTIVE IMMUNIZATION[1]

By G. G. Waters and P. J. Moloney

Abstract

C. novyi antitoxin injected in divided doses (total 500 units) over a period of 12 days was more effective in saving guinea pigs inoculated with spores of *Clostridium novyi* than was the same total amount of antitoxin given in one dose. Of the infected animals, 60 to 80% could be saved by treatment with divided doses of antitoxin up to 12 hr. after inoculation with spores. If, however, treatment was deferred for 24 hr., none of the animals could be saved. Preliminary results on active immunization with *novyi* toxoid indicate that a relatively high titer of antitoxin (in the order of 1μ per ml.) is required to protect guinea pigs against inoculation with *novyi* spores suspended in calcium chloride solution.

Introduction

Treatment, with antitoxin, of experimental gas gangrene induced in guinea pigs by *Clostridium novyi*, has been reported by others. Gordon and McLeod (2) found that *novyi* antitoxin was not effective as a curative agent in the guinea pig inoculated in a crushed leg muscle with a 24 hr. broth culture of *C. novyi*. The maximum total amount of antitoxin per guinea pig that Gordon and McLeod used was 3600 units. This was injected in two doses at four and seven hours after inoculation.

Klose and Schröer (3) claimed that mice inoculated with a dilution of a 24 hr. broth culture of *C. novyi* mixed with mucin survived when *novyi* antitoxin was injected three hours after inoculation of the broth culture. The amount of antitoxin administered to each mouse was 0.7 ml. serum containing 56 units of *novyi* antitoxin.

Klose and Schröer probably injected the antitoxin subcutaneously since other antitoxins studied by them and reported in the same paper were injected subcutaneously. However, in the case of *novyi* antitoxin, the text indicating the route of injection reads P.O. indicating, no doubt, per os. This is probably a misprint since antitoxin per os would almost certainly not have been effective.

Evans (1) induced gas gangrene in the guinea pigs by injecting a suspension of washed young organisms into the thigh of the animal three hours after an injection of calcium chloride had been given. He found that, with *C. novyi* infection, protection from death could be achieved only by the intravenous injection of large doses of antitoxin (in the order of 2500 units per animal) in an early stage of the disease. Animals could be saved up to 16 hr. but not 24 hr. after inoculation.

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Contribution from Connaught Medical Research Laboratories, University of Toronto, Toronto, Ont.

In this communication we are reporting some experiments on the treatment with antitoxin of experimental *C. novyi* infection in guinea pigs; in addition, we are presenting some preliminary observations on active immunization of guinea pigs against *C. novyi* infection.

Methods

C. novyi infection was induced in guinea pigs by the intramuscular injection of spores of *C. novyi*.

The spore suspension used was prepared as follows: Kidney meat broth (4) was seeded with *C. novyi* N21D and incubated at 38° C. for five days. The supernatant broth culture was separated from the precipitate of ground meat and centrifuged. The supernatant, after centrifuging, was drawn off and discarded, and the deposited microorganisms were washed by mixing with sterile physiological saline and again centrifuging. This process of washing was repeated three times. The washed organisms were then resuspended in a volume of saline equal to that of the supernatant broth from which they came, and left in the cold room at 5° to 8° C. for two days, after which they were heated at 70° C. for 15 min. and then stored in the cold room at 5° to 8° C. This spore suspension showed no noticeable loss in killing power when tested on guinea pigs after storage for one year.

The spore suspension alone, when injected intramuscularly and without trauma, was not lethal for guinea pigs. Death, however, as a result of gas gangrene infection, could be induced, just as with tetanus spores, by injecting spore suspension diluted with calcium chloride solution. For this purpose we found that calcium chloride in a concentration of 8% was suitable.

TABLE I

INTRAMUSCULAR INJECTIONS INTO GUINEA PIGS OF SPORES OF *C. novyi* IN CALCIUM CHLORIDE SOLUTION

Dilution	Number of animals living	Number of animals dead	% Survival
1/10,000	0	33	0
1/100,000	5	31	14
1/1,000,000	33	1	97

In Table I there is given a summary of the effect of intramuscular injection of the spore suspension described above, in 8% calcium chloride solution. The guinea pigs used weighed about 500 gm. each and each animal was injected intramuscularly with 0.5 ml. of the diluted suspension.

It will be noted that all the guinea pigs that received the 1/10,000 dilution and most of those that received the 1/100,000 dilution died.

The *novyi* antitoxin used throughout was derived from the blood plasma of horses that had been hyperimmunized by injections of *novyi* toxin. The

antitoxin in the plasma was purified and concentrated by a method involving peptic digestion.

Effect of Single and Multiple Doses of *novyi* Antitoxin

Preliminary experiments were carried out in which a single dose of antitoxin of varying strength, up to 500 units, was administered to guinea pigs at different times after inoculation with spores of *C. novyi*.

The administration of antitoxin rarely prevented death although the life of many of the animals was prolonged. As a result of this preliminary work, it was decided to set up an experiment in which the effect of antitoxin in single and divided doses might be compared. To this end, 180 guinea pigs of about 500 gm. each were injected in the thigh muscle with 0.5 ml. of a 1/10,000 dilution of spore suspension in 8% calcium chloride solution. At different times after the inoculation with spores, groups of the animals were injected intraperitoneally with *novyi* antitoxin. For example, with one group of 45 guinea pigs, each animal was injected with 500 units of antitoxin five hours after inoculation with spores; in a second group of 45 guinea pigs, each animal was injected, five hours after inoculation with spores, with 200 units of antitoxin followed by 50 units every 48 hr. until a total of 500 units had been injected.

TABLE II

CURATIVE ACTION OF ANTITOXIN FOLLOWING INJECTION OF SPORE SUSPENSION

Interval between inoculation of spores and administration of antitoxin (hours)	Survivals after:	
	500 units in one dose	200 units + 50 units × 6 over a period of 12 days
5	4/45 (9%)	27/45 (60%)
8	1/15 (7%)	9/15 (60%)
12	1/15 (7%)	12/15 (80%)
24	0/15 (0%)	0/15 (0%)

Results are summarized in Table II. In the table there is set forth the times of administration of the antitoxin following inoculation with spore suspension and the manner in which the antitoxin was given, that is, in single and divided doses, and also the number of guinea pigs in each group that survived.

It will be noted that 500 units of antitoxin injected in divided doses over a period of 12 days was more effective in saving the guinea pigs than was 500 units in one dose.

In order to obtain information regarding the antitoxin titers of the blood of guinea pigs that had been injected with spores of *C. novyi* followed by antitoxin, blood titrations were carried out on some of the guinea pigs of the group that were treated with antitoxin five hours after the injection of spores. In addition to titrating the blood of these animals, the bloods of other guinea

pigs that had not been inoculated with spores but that had received antitoxin alone were also titrated. Results are summarized in Table III. Each result recorded in the table represents units of antitoxin on a pool of equal amounts of sera from three guinea pigs.

TABLE III

C. novyi ANTITOXIN TITERS FOLLOWING INTRAPERITONEAL INJECTION OF ANTITOXIN

Days after injection	Spores injected five hours previous to antitoxin		No spores injected	
	500 units in one dose	200 units* + 50 units × 6	500 units in one dose	200 units* + 50 units × 6
1	8 μ/ml.	2.6 μ/ml.	8 μ/ml.	2.6 μ/ml.
4	2.6 "	0.9 "	2.6 "	2.6 "
6	0.9 "	0.7 "	2.0 "	2.0 "
8	0.7 "	0.9 "	0.7 "	2.0 "
10	<0.15 "	0.7 "	0.2 "	0.9 "
13	<0.15 "	0.3 "	<0.15 "	0.9 "
15	<0.15 "	0.7 "	<0.15 "	0.7 "

* *Initial dose 200 units, followed by six doses of 50 units each every 48 hr.*

In the guinea pigs that received a single dose of antitoxin there was less than 0.15 units (lower limit of test) from the 10th to the 13th day on, whereas, as one might expect, antitoxin was present in measurable amounts to the 15th day in the guinea pigs that received the same total amount of antitoxin in divided doses.

Note on Immunization with *novyi* Toxoid

When the study of the possible curative action of *novyi* antitoxin was in progress, work was also undertaken on active immunization with *novyi* toxoid. Only preliminary observations were made and these we are reporting here.

C. novyi toxin (broth filtrate) was detoxified, as in the case of diphtheria and tetanus, by the addition of formalin and storing at 34° to 36° C. until detoxification was complete.

Three doses of the detoxified toxin were injected subcutaneously into each of 22 guinea pigs of about 300 gm. weight, with an interval of two weeks between the first and second injections and three weeks between the second and third. Twenty-five days after the third injection the animals were bled, and five days later each was inoculated intramuscularly with 0.5 ml. of 10^4 dilution of spore suspension.

In Table IV there is summarized antitoxin titers of the blood sera and deaths.

Blood was drawn from the three surviving guinea pigs 4 days and 12 days after inoculation and the sera were titrated for *novyi* antitoxin. In addition, the sera from bloods drawn before inoculation were retitrated at closer intervals (Table V).

TABLE IV

C. novyi TOXOID

ANTITOXIN TITERS AND EFFECT OF SPORE INOCULATION

Guinea pig No.	Antitoxin titer before spore inoculation	Record of deaths					
		Days after spore inoculation					
		1	2	3	4	5	6
3508	<0.06 μ/ml.	−	+				
808	"	−	+				
3980	"	−	+				
3595	"	−	+				
3663	"	−	−	+			
3735	"	−	−	+			
3978	"	−	−	+			
3478	>0.06 <0.3	−	−	+			
3744	"	−	−	+			
509	"	−	−	+			
546	"	−	−	+			
3820	"	−	−	−	+		
158	"	−	−	−	+		
355	"	−	−	−	+		
487	"	−	−	−	+		
3486	>0.3 <1.5	−	+				
3964	"	−	−	−	−	+	
3440	"	−	−	−	−	+	
3519	"	−	−	−	−	−	+
552	"	−	−	−	−	−	−
830	"	−	−	−	−	−	−
998	"	−	−	−	−	−	−

TABLE V

EFFECT OF SPORE INOCULATION ON ANTITOXIN TITER

Guinea pig No.	Antitoxin in blood serum		
	Before inoculation	Four days after inoculation	Twelve days after inoculation
552	1.2 μ/ml.	0.6 μ/ml.	>1.2 μ/ml.
830	0.6 "	0.3 "	>1.2 "
998	0.6 "	0.4 "	>1.2 "

Results in Table V show that the inoculation with spores caused a preliminary drop in antitoxin followed by a rise.

Discussion

The reason for more effective treatment with repeated doses of antitoxin as compared with treatment with the same total amount of antitoxin in a single dose, is no doubt, that the former method ensures effective concentrations of antitoxin (in the order of 1μ per ml.) in the infected animals for a longer period than does the latter.

If one wishes to compare results recorded here with the results of others, it is of course necessary to bear in mind that besides possible differences in strain of *C. novyi* used, there were differences in experimental method. Gordon and McLeod infected guinea pigs by injecting a fresh culture of *C. novyi* into a crushed muscle and treating the animals by administering antitoxin (probably intramuscularly) near the site of infection. The treatment was not successful. Gordon and McLeod were, however, able to prevent infection with *C. novyi* in guinea pigs by administering antitoxin before inoculation. Evans infected guinea pigs by injecting washed organisms from a fresh broth culture into muscle that had previously been injected with a solution of calcium chloride. Antitoxin was administered intravenously and in the early stages of the disease treatment was successful using a single relatively large dose of antitoxin.

Our results are in accord with those of the authors just cited, namely, that antitoxin is effective in controlling experimental infection with *C. novyi*, at least as a preventive agent.

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STUDIES ON THE EFFECT OF SOME AROMATIC AMIDINES AND OTHER CHEMICAL COMPOUNDS ON THE GROWTH OF INFLUENZA VIRUS IN THE EMBRYONATED EGG

By Laurella McClelland and C. E. van Rooyen

Abstract

The effect of chemicals and biological substances on the growth of influenza virus in the embryonated egg is of interest as a fundamental study and as a means of screening substances for their possible chemotherapeutic activities. This work was done to investigate the activity of some heretofore unreported substances. Most chemicals proved to have no effect on the growth of influenza virus in the developing egg, but one group, the aromatic amidines, and hexamidine in particular, was found to possess some inhibitory action. It was an *in vivo* rather than an *in vitro* effect; it was dependent on the route of inoculation and the amount of drug administered; and the activity was exhibited when the introduction of the drug was delayed for as long as 16 hr. after inoculation of the eggs with influenza virus. Preliminary tests have failed to demonstrate any protection of mice infected with influenza and treated with hexamidine.

The inhibitory action of organic and inorganic chemical substances on the growth of viruses in chicken embryos has attracted much attention. Many compounds have been tested for evidence of possible activity against influenza and other viruses in embryonated eggs and experimental animals, but relatively few have been noted to possess antiviral effect (2, 9, 3). Among the few substances that have been reported to show inhibitory effects are nitroakridin (5); chloromycetin (13); apple pectins and other polysaccharides (6). Certain bacterial extracts have also been shown to be active according to Horsfall and McCarty (8).

During the course of our experiments on influenza virus, we have examined a selection of chemical compounds for their inhibitory action, but the bulk of these have proved to be inactive. The present report includes all results and describes in detail some findings obtained with one member of the aromatic amidine series, namely hexamidine, 4 : 4′-diamidinodiphenoxyhexane diisethionate.

The aromatic amidines have been extensively studied by other workers in connection with the treatment of trypanosomiasis and leishmaniasis. Past investigations have covered a variety of aspects such as the effect of these drugs on human patients, laboratory animals, *in vitro* test tube experiments, and inquiries into their complex pharmacological and metabolic behavior (11, 1, 7, 4, 15, 10, 14).

Materials and Methods

Fertile eggs previously incubated for 11 days were employed throughout in the experiments. The seed virus used was from a stock of frozen allantoic fluid from eggs infected with influenza virus type A, PR8 strain.

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Contribution from the Connaught Medical Research Laboratories, University of Toronto, Toronto, Ont. This work was aided by a grant from the National Research Council of Canada.

First, the toxicity of the various drugs to embryos was ascertained. Freshly prepared solutions of the drug in a suitable solvent were inoculated in eggs via the chorioallantoic and yolk sac routes. This procedure was carried out in order to test drugs at an optimum dosage level. To demonstrate the method used, the experimental data on one drug are given in detail as follows. It was found in the case of hexamidine that it was relatively toxic to embryos. When 2 mgm. was injected into the allantoic cavity all the embryos died. When the amount of drug was decreased the number of surviving embryos was increased. In preliminary experiments on small groups of eggs, the average percentage mortality at 1.0 mgm. was 90%; at 0.5 mgm., 70%; at 0.25 mgm., 10%; and at 0.125 mgm., 7%. A test dose of 0.25 mgm. was selected as an amount that did not kill a proportionately high number of embryos as compared to the number that usually die as the result of any injection procedures. Similarly, hexamidine was found to be toxic when administered via the yolk sac route. In this instance, however, a greater amount of drug was required to produce the same percentage mortality and a dose of 0.5 mgm. was selected as the test dose.

Next, influenza virus was injected into the allantoic cavity and this was followed by the administration of the selected dose of drug, either into the same cavity or into the yolk sac. Inoculated eggs were incubated at 36° C. for 44 to 46 hr.; the allantoic fluid was aspirated and tested for evidence of influenza virus by examining the fluid of each egg for its ability to agglutinate fowl red blood cells; thereafter, aliquot volumes of positive fluids were pooled and the haemagglutinin titer of the combined fluids was determined using a modification of the method of Miller and Stanley (12). Hereafter this determination is referred to as C.C.A. (chicken cell agglutination) titer.

Preliminary screening tests showed that there was no evidence of any effect on virus growth when most of the drugs were tested in the manner described above. However, with some of the amidines, and with hexamidine in particular, there was some effect demonstrated when the drug was introduced into the allantoic cavity. Since it may be of interest and assistance to other workers in this field, we have recorded in Table I a list of selected chemicals tried. The data include the tolerated dose of each drug with the route of inoculation and the effect on virus growth. The remainder of the paper deals with the experimental work that was carried out in an effort to evaluate the antagonistic effect shown by hexamidine.

Experiment 1

This was designed to determine whether the action of the drug was an *in vivo* effect upon the growth of influenza virus in the embryo or whether it was due to interference with haemagglutination *in vitro*. Our results showed that hexamidine, in amounts equivalent to those that might be found in the allantoic fluid of drug treated eggs in the present work, when added to the mixture of PR8 infected allantoic fluid and fowl red blood cells did not alter the end point of the titration.

We considered also the possibility of the formation of a metabolic product in drug treated eggs that might have interfered with haemagglutination and so accounted for the fall in haemagglutinin titer. Normal embryonated eggs

TABLE I

Chemical tested	Dose in mgm. and route of inoculation	Result
Benzpyrene	1 (A)* & (YS)†	Negative
Methyl benzanthracene	0.25 (A) & (YS)	Negative
Ethyl benzanthracene	0.25 (A) & (YS)	Negative
Propyl benzanthracene	0.25 (A) & (YS)	Negative
Butyl benzanthracene	0.25 (A) & (YS)	Negative
Amyl benzanthracene	0.25 (A) & (YS)	Negative
Hexyl benzanthracene	0.25 (A) & (YS)	Negative
Dibenzanthracene	0.5 (A) & (YS)	Negative
Thyroxin	0.0003 (A)	Negative
Thiouracil	1.0 (A)	Negative
Vitamin B6	2.0 (A) & (YS)	Negative
p-Aminobenzoic acid	2.0 (A) & (YS)	Negative
Stilboestrol	1.0 (A) & (YS)	Negative
Hexoestrol	0.5 (A) & (YS)	Negative
Propylene glycol	0.02 (A)	Negative
Potassium cyanide	0.25 (A) & (YS)	Negative
Copper sulphate	0.5 (A) and 0.1 (YS)	Negative
Silver nitrate	5.0 (A) & (YS)	Negative
Sodium fluoride	2.0 (A) & (YS)	Negative
Sodium arsenite	0.2 (A) and 0.1 (YS)	Negative
Phenamidine	2.0 (A)	Slight
Stilbamidine	10.0 (A)	Slight
Hydroxystilbamidine	10.0 (A)	Slight
Propamidine	2.0 (A)	Negative
Dibrompropamidine	0.75 (A)	Negative
Pentamidine	0.3 (A)	Active
Hexamidine	0.5 (A)	Active
Heptamidine	0.2 (A)	Active
Octamidine	0.1 (A)	Slight

* *(A) Allantoic.*
† *(YS) Yolk sac.*

were injected with 0.25 mgm. of drug and after 48 hr. incubation the allantoic fluid was pooled and tested for the presence of agglutinin-inhibiting substances, but none were found. The control titrations were made using normal allantoic fluid.

The above experiments showed that the low haemagglutinin titer found in hexamidine treated eggs was not due to the direct action of hexamidine on red cell agglutination or to the presence of antagonistic substances in the allantoic fluid.

Experiment 2

This was designed to find whether hexamidine exhibited any direct *in vitro* antiviral activity.

To a 10^{-3} dilution of PR8 virus in Ringer's solution, enough drug was added to make a final concentration of 0.25 mgm. per ml. The mixture was incubated at 37° C. for two hours and from it were made five 10-fold dilutions up to 10^{-8}. Eggs were inoculated with each dilution to determine the 50% infective dose (ID_{50}) and to compare the results of red cell agglutination titers with eggs that had received an identical range of virus dilutions prepared as above but without hexamidine. The results (Table II) indicated that although the drug was not comparable in effect to that of an antiseptic such as formalin, there was some *in vitro* effect as evidenced by the shift in the ID_{50}.

TABLE II

In vitro EFFECT OF 0.25 MGM. HEXAMIDINE ON INFLUENZA VIRUS AS DEMONSTRATED BY INFLUENCE ON INFECTIVITY TO EMBRYONATED EGGS AND C.C.A. TITER

Virus dilution*	First test				Second test			
	Ratio of positive eggs to total eggs tested		C.C.A. titer of positive eggs		Ratio of positive eggs to total eggs tested		C.C.A. titer of positive eggs	
	Drug treated inoculum	Control inoculum	Drug treated inoculum	Control inoculum	Drug treated inoculum	Control inoculum	Drug treated inoculum	Control inoculum
10^{-4}	6/10	8/10	1/676	1/708	10/10	9/10	1/407	1/432
10^{-5}	4/9	9/10	1/624	1/1024	9/9	9/9	1/394	1/468
10^{-6}	3/9	7/9	1/1024	1/1024	7/9	9/10	1/380	1/537
10^{-7}	0/10	4/9	—	1/841	2/8	4/8	1/403	1/474
10^{-8}	0/10	0/9	—	—	0/9	1/10	—	1/339

* *Dose of virus dilution was 0.2 ml. per egg.*

ID_{50} of drug treated inoculum as compared to control inoculum in first test, $10^{-4.8}$ to $10^{-5.4}$; in second test, $10^{-6.5}$ to 10^{-7}.

Experiment 3

Table III shows the effect of the administration of diminishing doses of drug ranging from 1 mgm. to 0.015 mgm. to eggs previously inoculated with a fixed amount of virus (0.2 ml. of a 10^{-6} dilution that by previous titrations was shown to be between 1 and 10 ID_{50}). The results would indicate that a direct correlation existed between the amount of drug introduced and the resulting haemagglutinin titer of the allantoic fluid.

Experiment 4

In previous tests it was shown that the drug was able to inhibit the growth of influenza virus when eggs were inoculated with 1 to 10 ID_{50} doses of virus. In the present test, it was shown that the drug was also potent against still higher doses of virus. Table IV presents the results. It should be noted

TABLE III

EFFECTS OF DECREASING AMOUNTS OF HEXAMIDINE ON THE PRESENCE OF VIRUS IN THE ALLANTOIC FLUID AND ON THE C.C.A. TITER OF POOLED POSITIVE FLUIDS: TWO DIFFERENT EXPERIMENTS ARE RECORDED

Test No.	Dose of drug in mgm.	No. of eggs inoculated	Ratio of positive eggs to total eggs tested	C.C.A. titer of pooled positive fluids
A	1.0	10	0/3*	—
	0.5	10	1/5	1/44
	0.25	9	7/9	1/93
	0.125	10	7/8	1/299
	0.06	10	8/9	1/417
	0.03	10	9/9	1/367
	0.015	9	8/9	1/549
	0 (Control)	9	7/8	1/512
B	0.25	12	11/12	1/128
	0.125	12	12/12	1/221
	0.06	12	12/12	1/309
	0.03	11	11/11	1/363
	0 (Control)	11	9/11	1/550

* *The toxicity of the drug can be gauged by comparing the number of surviving eggs tested with the number of eggs inoculated.*

TABLE IV

EFFECT OF 0.25 MGM. HEXAMIDINE ON INCREASING DOSES OF VIRUS, TESTED *in vivo*

Dose of inoculum in ID_{50}'s	Ratio of positive eggs to total eggs tested		C.C.A. titer of pooled positive fluids	
	Drug treated	Control	Drug treated	Control
1	0/9	3/10	—	1/256
10	3/6	9/10	1/38	1/367
100	8/9	8/9	1/73	1/335
1000	9/9	9/9	1/93	1/320
10,000	8/9	9/9	1/86	1/335

that the action of the drug was most pronounced on small dosage of virus. Thus, when small amounts of virus were administered to the egg, both the number of eggs infected and the corresponding C.C.A. titer were markedly reduced. On the other hand, when large doses of virus were inoculated, the effect was reflected only in the diminished C.C.A. titers of positive fluids.

Experiment 5

This series was planned to explore the effect of administering hexamidine at time intervals before and after infection with virus.

Table V contains a summary of findings. The results show that the drug is active if administered either two and one-half hours before introduction of virus or as late as 16 hr. after virus inoculation.

TABLE V

EFFECT OF ADMINISTERING HEXAMIDINE BEFORE AND AFTER INOCULATION OF VIRUS

Test No.	Interval between injection of drug and inoculation of virus*	Number of eggs inoculated	Ratio of positive eggs to total eggs tested	C.C.A. titer of pooled positive fluids
1	Drug 2½ hr. before virus	19	17/18	1/64
	Drug within 5 min.	19	15/18	1/82
	Drug 5 hr. after virus	20	15/15	1/59
	Control	19	17/18	1/473
2	Drug 2½ hr. before virus	16	13/15	1/116
	Drug within 5 min.	16	14/14	1/94
	Drug 5 hr. after virus	16	12/13	1/170
	Control (for above)	16	12/15	1/512
	Drug 16 hr. after virus	16	13/14	1/146
	Control (for 16 hr. test)	16	13/16	1/512

** Dose of drug: 0.25 mgm. per egg; dose of virus: 0.2 ml. of 10^{-6} dilution of virus. Both inoculations made into allantoic cavity.*

Experiment 6

This experiment was an attempt to determine the influence of hexamidine on the normal growth curve of influenza virus in the embryonated egg. Eggs treated with 0.25 mgm. of hexamidine and untreated control eggs were inoculated with 0.2 ml. of a 10^{-6} virus dilution (1 to 10 ID_{50}) and were incubated. Throughout the next 48 hr., samples of allantoic fluid were aspirated at four hourly intervals and were tested for presence of virus. Control eggs first showed red cell agglutination after 28 hr. incubation, but in drug treated eggs, such response was delayed for as long as 40 hr. In order to ascertain the extent of delay in the multiplication of virus, a second experiment was performed. Three groups of drug treated eggs were inoculated with influenza virus and incubated for 30, 48, and 72 hr. respectively. Thereafter, the eggs were removed from the incubator, chilled by being placed in ice cold water, and the allantoic fluid was aspirated. This was tested for the presence of virus and the C.C.A. titer was measured. Appropriate controls were incorporated. Findings are summarized in Table VI. It should be noted that notwithstanding the prolonged incubation of eggs for an additional 24 hr. more than the usual 48 hr., the C.C.A. titer of the drug treated eggs remained low as compared to the titer of the control eggs.

Experiment 7

It is recognized that the infectivity of allantoic fluid containing viable virus is roughly proportional to the chicken cell agglutination titer of that fluid, but it is also known that fluids that do not produce agglutination of red blood cells may be infective. Therefore, an embryo titration enables one to estimate more precisely the amount of viable virus present in any preparation. To determine the extent of the diminution of the amount of virus in fluids from drug treated eggs, the fluids were titrated in eggs and the 50% end points

TABLE VI

EFFECT OF PROLONGED INCUBATION ON VIRUS CONTENT IN ALLANTOIC FLUID OF HEXAMIDINE TREATED AND UNTREATED EGGS

Hours of incubation	Ratio of positive eggs to total eggs tested		C.C.A. titer of pooled positive fluids	
	Treated*	Control†	Treated	Control
30	0/11	6/13	—	1/226
48	7/12	10/13	1/61	1/462
72	12/12	9/12	1/54	1/407

* *Treated eggs received 0.25 mgm. hexamidine and 0.2 ml. of a 10^{-6} dilution PR8 influenza simultaneously.*

† *Control eggs received 0.2 ml. of a 10^{-6} dilution PR8 influenza.*

were estimated. The percentage loss in infectivity was then calculated, taking as 100% the end point titer of the fluids of infected, untreated eggs. Results of such titrations and the calculations of virus loss are presented in Table VII.

TABLE VII

COMPARISON OF C.C.A. TITER WITH ID_{50} OF ALLANTOIC FLUIDS OF UNTREATED (CONTROL) AND DRUG TREATED EGGS

Untreated or treated	C.C.A. titer of pooled fluids	ID_{50} of pooled fluids	Percentage virus present	Percentage diminution of virus
U*	1/316	9.280	100	
T†	> 1/2	6.822	0.35	99.65
U	1/330	9.78	100	
T	> 1/2	8.48	5.01	95
U	1/330	9.44	100	
T	> 1/2	8.5	10.14	89.86
U	1/330	9.63	100	
T	> 1/2	7.75	1.318	98.682
U	1/468	9.50	100	
T	> 1/2	7.423	0.83	99.17

* *Untreated.*

† *Treated.*

Discussion

Although many of the chemicals tested were ineffective against influenza virus, the most active drug encountered was 4 : 4′-diamidinodiphenoxyhexane diisethionate (hexamidine), a member of the aromatic amidine group of compounds, the pharmacological action of which in man and animals has already been studied.

Our tests have shown that the toxicity of the drug to chicken embryos varied according to the route of administration, being more toxic by the allantoic route than by the yolk sac. Likewise, the activity of the drug against influenza virus depended on the route of inoculation, being active by the allantoic route only.

Inquiry into the time factor showed that the drug was able to inhibit the development of virus when it was administered as long as 16 hr. after introduction of virus. Further work along these lines may yield information concerning the mechanism by which hexamidine interferes with the multiplication of influenza virus in the embryonated egg.

Experiments indicated that the neutralizing effect of hexamidine on influenza virus was best shown when an adequate amount of drug was administered in the presence of a small dosage of virus, although some action was also evident with larger virus and smaller drug dosage. This effect is exhibited by most chemotherapeutic agents, i.e., an adequate drug dosage must be administered to cope with the infecting agent, and it is important to remember this when chemotherapeutic screening tests are performed with eggs.

The loss of infectivity of low C.C.A. titer fluids from eggs that had been treated presents a more quantitative measure of virus inhibition. Although one was disappointed not to find complete absence of virus in fluids that showed no chicken cell agglutination, there was found to be a loss of from 99.65 to 89.86% of virus in such fluids. In using chicken cell agglutination in screening tests it would appear advisable to employ it as an indicator only, and, as the final criterion of inhibition of virus growth, to rely on the more sensitive test, that of the infectivity of the fluid in question.

Preliminary experiments on the chemotherapeutic effect of hexamidine when administered subcutaneously to mice have failed to demonstrate any protection against infection with influenza. Although this is disappointing it should be remembered that the mechanism of virus multiplication is not known and that although a compound may inhibit growth of a virus in chick embryo protein, it does not necessarily follow that the same response should be expected from adult mouse tissue.

Acknowledgments

The authors are indebted to Dr. A. J. Ewins, of Messrs. May and Baker, London, for a generous supply of amidines, also to Dr. Alexander Haddow, of the Royal Cancer Hospital, London, for carcinogenic compounds. We owe our thanks to Miss E. K. Campbell and Miss Greta Nicolson for their technical assistance, and to Dr. Angus F. Graham for much helpful criticism.

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AN ELECTRIC CAUTER AS A STERILITY AID IN INOCULATION OF EGGS[1]

By N. A. Labzoffsky

Abstract

An electric cauter for sterilization of the opening through which embryonated egg is inoculated is described. The apparatus consists of a transformer, a rheostat, two leads, and a nichrome wire tip; the latter serves as a hot point. The temperature that develops at the tip is approximately 1000° C. and, therefore, instantaneous application of the hot point to the hole in the egg shell results in absolute sterilization of the area.

It is generally known among laboratory workers that application of iodine or any other disinfectant to the shell, to sterilize the opening through which the embryonated egg is inoculated, does not ensure the desired sterility at the site of inoculation and may result in a significant percentage of contaminations when a large number of eggs is being inoculated.

To overcome this, a simple and highly efficient electric cauter has been employed in our laboratory, which reduces contamination of the eggs during inoculation to practically nil. The cauter, of course, is suitable only for the air sac route inoculation.

The apparatus consists of a transformer (110 v. primary, 6 amp., 2.5 secondary), a rheostat (100 ohm, 25 to 50 w.), two leads, and a tip. The construction of the apparatus can be followed in detail by reference to Fig. 1.

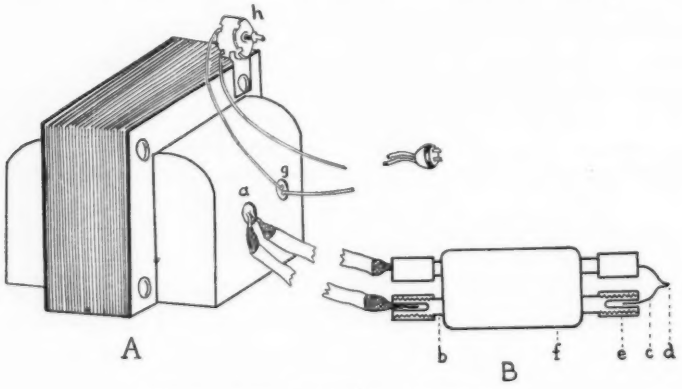

Fig. 1. *A = Transformer. B = Cauter.*

Each lead, preferably flexible braided electrical cable of low resistance (known as "tinned copper shielding"), is approximately 3 ft. long and is

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Contribution from the Virus Section, Division of Laboratories, Ontario Department of Public Health, Toronto, Ont.

incased in a rubber tubing of a suitable diameter for insulation. One end of each lead is connected to the secondary winding of the transformer (Fig. 1 A, *a*) and the other end to a brass rod (B, *b*). The brass rods are approximately 3/16 in. in diameter and 3 in. long. The free ends of the brass rods are connected to each other by nichrome wire (*c*), which serves as a hot point. The nichrome wire, gauge 22 B & S, is about 1 in. long and bent in the middle at an acute angle to form a sharp point (*d*). To facilitate the assembly of the apparatus, as well as to provide a proper contact, the ends of the rods are threaded and slotted to a depth of $\frac{1}{2}$ in. Screw type sleeves (*e*) go over the ends of the rods so that when the end of the lead or the nichrome wire is inserted into the slot, a low resistance connection is obtained by tightly screwing the sleeve. The rods are $\frac{1}{4}$ in. or so apart and are embedded in plaster of Paris (*f*).

The primary winding of the transformer (*g*) is connected to an electric cord with a plug and rheostat (*h*), which is mounted on the transformer.

For operation, the apparatus is plugged into an electric outlet and the temperature of the point is adjusted to the first visible white heat with the rheostat. The eggs are drilled in the usual manner with some sharp instrument without breaking the shell membrane, and the opening is cauterized by touching it with the hot point. The temperature that develops at the point is approximately 1000° C. and therefore instantaneous application of the point to the opening ensures absolute sterilization of the area.

By using tungsten wire of a suitable gauge, instead of nichrome wire, it may be possible to obtain a sufficiently high temperature at the point to burn holes through the shell without preliminary drilling.

An electrically heated wax pencil for sealing inoculated eggs, which we found to be a very convenient and time-saving device, has been described elsewhere (1).

Reference

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A SIMPLE GAS ANALYZER[1]

By F. E. J. Fry

WITH AN APPENDIX

By A. C. Burton and O. G. Edholm

Abstract

The gas analysis apparatus described is similar to an enlarged version of Krogh's microapparatus. It consists of a 1 cc. burette calibrated in one-hundredths, enclosed in a water jacket joined above to a glass funnel through a short capillary and below to a mercury reservoir. The absorbing solutions are placed in the glass funnel above, and the gas sample is injected through a needle inserted into a rubber cap on a side arm of the mercury reservoir.

The apparatus is simple in design and in use, is easy to clean, and the cost is low. Accuracy is approximately ± 0.5%. A modified analyzer has been used by students with satisfactory results.

Introduction

Analyzers of the type described here were first constructed early in 1942 and used extensively by investigators of the Royal Canadian Air Force in the study of oxygen apparatus. These analyses were routinely carried out in decompression chambers at altitudes up to 40,000 ft. and occasionally in aircraft as well as under more conventional laboratory conditions. The apparatus proved extremely convenient and reliable and its operation is so simple that an inexperienced person can produce fair results after one or two trials. An analysis requires about five minutes and if care is taken to allow for complete drainage, the probable error of a single determination is about 0.05% when the gas measured is 20% of the total volume of the sample.

Construction

Working drawings for the construction of the analyzer are given in Fig. 1. It consists of a calibrated glass tube of about 3 mm. bore and 25 cm. long, with a capillary of about 1 mm. bore and 5 cm. long attached to one end and a bulb of a little more than 5 cc. capacity blown on the other. A cup of about 2 to 3 cc. capacity is blown on the free end of the capillary. Two short arms are fused to the 5 cc. bulb, one of these being opposite the calibrated tube and the other near the first arm and making an angle of about 45° with it. The arm in line with the capillary is about 12 mm. in diameter and 3 cm. long, the side arm has a diameter of about 6 mm. and is less than 1 cm. long. Both are flared at the open end.

The zero mark for the calibrated section is arranged to come about one-third of the way up the 1 mm. capillary. The total volume of the calibrated

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Contribution from the Department of Zoology, University of Toronto, Toronto, Ont. and the Department of Physiology, University of Western Ontario, London, Ont.

portion is 1 cc. and the graduations are one-hundredths. This portion can be conveniently made from a 1 cc. pipette cut off at the 0.01 cc. mark for attachment to the capillary. It is then necessary only to determine the position of the zero mark on the capillary to complete the calibration.

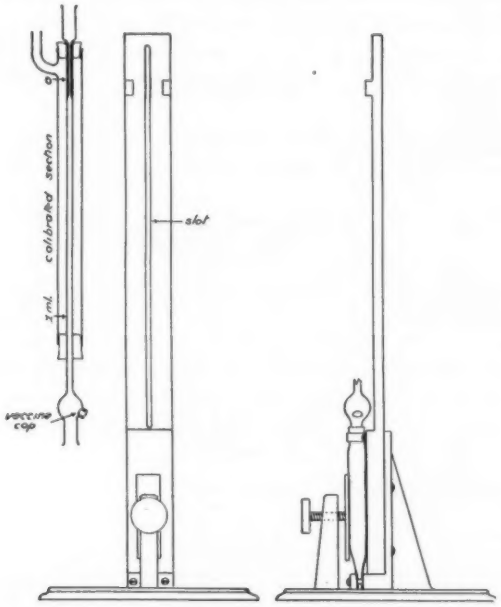

FIG. 1. *Drawing of apparatus. The glass parts are shown on the left. The side arm on the water jacket can be replaced by making an opening in the upper cork. The stand and reservoir are shown in two aspects. The base is of metal, the upright is wooden.*

The calibrated tube is provided with a water jacket. It is convenient to slip a rubber stopper in place around the pipette before attaching the bulb to close the lower end of the water jacket. A split stopper can be used at the upper end. If desired, the jacket can be blown integrally with the pipette.

A piece of medium wall rubber tubing about 12 cm. long is bound tightly to the terminal arm on the bulb and the apparatus is mounted vertically on a suitable board with the cup uppermost. Synthetic rubber tubing should not be used. The free end of the rubber tube is closed by a clamp on the base. A suitable screw adjustment is provided for flattening the tube with a metal tongue.

The side arm of the bulb is provided with a rubber cap soft enough to allow a No. 26 or 27 hypodermic needle to penetrate it and thick enough to prevent more than a droplet of mercury from forcing its way through the puncture on the withdrawal of the needle. A vaccine cap is convenient for this purpose.

Operation

Directions for operation, designed as a laboratory sheet for students, are given in the appendix.

The solutions used consist of $N/10$ potassium hydroxide for the absorption of carbon dioxide and sodium anthraquinone-β-sulphonate for the absorption of oxygen. This last solution is prepared from a mixture of one part sodium anthraquinone-β-sulphonate to five parts of sodium hydrosulphite dissolved in $N/10$ potassium hydroxide. The solution should be prepared fresh at least every day.

Latterly, indigo carmine has been substituted for the anthraquinone, using the same concentration. It has the advantage of being more stable.

Acknowledgments

The original apparatus was constructed in No. 1 Clinical Investigation Unit of the Royal Canadian Air Force, the funds being made available through the Associate Committee of Aviation Medicine of the National Research Council of Canada. The apparatus, as no doubt will be recognized, is more or less an enlarged version of Krogh's (1) microapparatus. The hypodermic method of introducing the sample was learned from Dr. P. F. Scholander of Swarthmore College.

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APPENDIX

Use of the Fry Analyzer for Student Classes

The apparatus designed by Dr. Fry has been used in the practical physiology class for medical students with satisfactory results.

One advantage of this apparatus is the low cost. The water jacket was constructed without internal seals or side arm, being closed by rubber corks above and below. The upper cork has a split in it for filling the jacket and to allow for changes in volume.

TABLE I

COST OF STUDENT APPARATUS

Total cost for:	
24 Analyzers	$212.50
Each	8.85
Detailed cost for 24 analyzers:	
Metal parts	24.80
Glass parts	31.00
Wood	10.00
Labor	
Wood work	31.00
Glass blowing	60.00
Assembly	55.70

Twenty-five sets were constructed at a cost of $8.85 for each analyzer (excluding mercury). The work was carried out in the University workshops and includes full charge for labor. Table I gives the details of the expenses.

The results of gas analyses by students at their first trial are shown in Fig. 2.

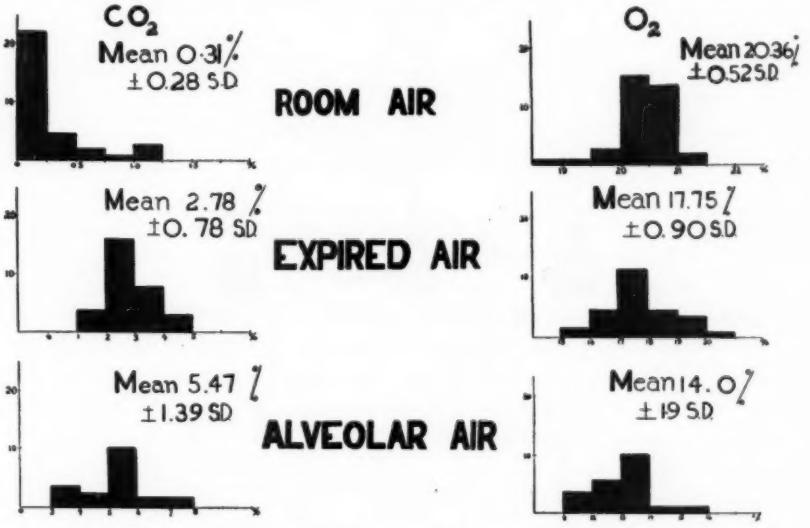

FIG. 2. *Results obtained by students at first trial. All students analyzed the same room air, and also the same expired air, which was collected in a Douglas Bag. A check analysis showed the composition was: carbon dioxide 2.9%; oxygen 17.6%. The alveolar air samples were collected individually using a Haldane tube. The material analyzed might therefore differ considerably. Error in oxygen determination is greater than carbon dioxide determination as two operations are involved.*

It should be emphasized that these results were obtained after a brief demonstration and were the first gas analyses performed by the students. Reproduced below is the instruction sheet issued to the class. Fig. 3 shows the diagrams issued to illustrate the technique.

The advantages of this apparatus for student use are simplicity, absence of taps, ease of cleaning, and low cost. The main source of error in student hands is the risk of losing part of the gas sample by too sudden movements of the screw controlling the mercury level.

The use of the analyzer in classwork means that the student does not have to spend an undue amount of time in learning technique and can obtain valid results in experiments on respiration with comparative ease.

Instruction Sheet

THE ANALYSIS OF RESPIRATORY GASES

The apparatus used is known as the Fry Analyzer, after the name of the inventor. It is one of many types of apparatus, but has the advantage of

relative simplicity. The principle of this, as with other types, is that a sample of gas is introduced and its volume (A) accurately measured. The carbon dioxide is absorbed by bringing the gas into contact with sodium hydroxide, and the volume (B) of gas left is measured—the difference of $A - B$ = volume of carbon dioxide. Oxygen is then absorbed with

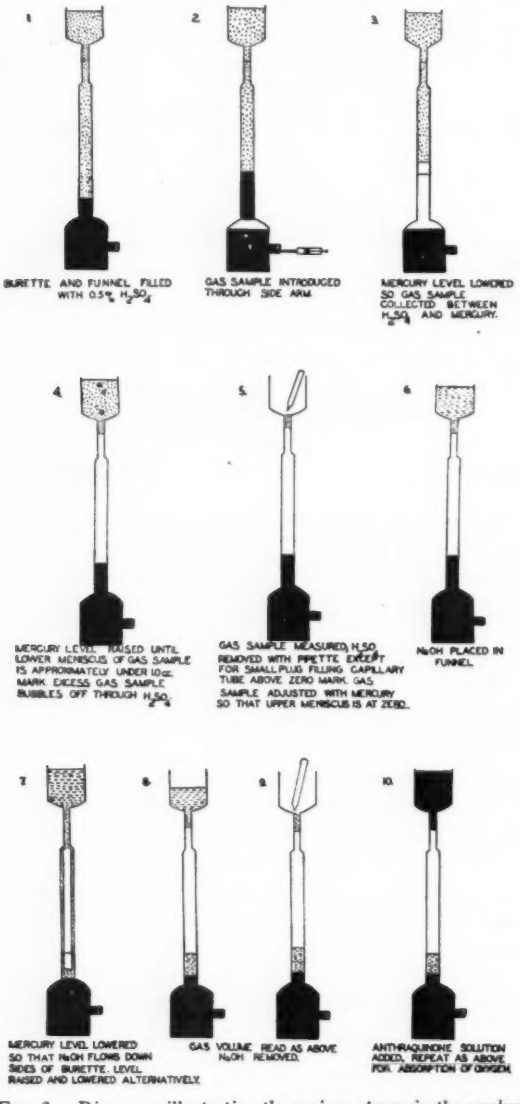
1
BURETTE AND FUNNEL FILLED WITH 0.5% H_2SO_4
2
GAS SAMPLE INTRODUCED THROUGH SIDE ARM
3
MERCURY LEVEL LOWERED SO GAS SAMPLE COLLECTED BETWEEN H_2SO_4 AND MERCURY.
4
MERCURY LEVEL RAISED UNTIL LOWER MENISCUS OF GAS SAMPLE IS APPROXIMATELY UNDER 10cc. MARK. EXCESS GAS SAMPLE BUBBLES OFF THROUGH H_2SO_4
5
GAS SAMPLE MEASURED, H_2SO_4 REMOVED WITH PIPETTE EXCEPT FOR SMALL PLUG FILLING CAPILLARY TUBE ABOVE ZERO MARK. GAS SAMPLE ADJUSTED WITH MERCURY SO THAT UPPER MENISCUS IS AT ZERO.
6
NaOH PLACED IN FUNNEL
7
MERCURY LEVEL LOWERED SO THAT NaOH FLOWS DOWN SIDES OF BURETTE. LEVEL RAISED AND LOWERED ALTERNATIVELY.
8
GAS VOLUME READ AS ABOVE NaOH REMOVED.
9
10
ANTHRAQUINONE SOLUTION ADDED, REPEAT AS ABOVE, FOR ABSORPTION OF OXYGEN.

FIG. 3. *Diagrams illustrating the various stages in the analysis.*

anthraquinone and the final volume (C) measured, $B - C$ = volume of oxygen. The remainder is assumed to consist of nitrogen. The rare gases are not considered.

Directions

1. Raise mercury level by turning screw at base until it reaches junction of burette and funnel.

2. Pipette acid water (0.5% sulphuric acid) into the funnel at the top of the apparatus.

3. Lower mercury level by means of screw so that funnel and burette are filled with acid, and mercury stands 1 cm. approximately above the bulb of the burette.

4. Introduction of gas. The first analysis will be of room air. A hypodermic syringe is filled with air by repeated filling and emptying so that a representative sample is obtained. From this point on, the plunger must not be withdrawn. A needle is put on the syringe and enough gas expressed to wash out bore of needle. The needle is pushed through the small rubber cap on the side of the burette bulb and 2 to 3 cc. of gas introduced. The mercury will rise in the burette trapping the gas in the bulb.

5. Lower the mercury in the burette by means of the screw until it has all come back into the reservoir.

6. Measurement of sample. Raise the gas sample into the burette by means of the screw until the water meniscus is approximately at the 0.95 cc. mark. Excess gas will escape through the funnel. Remove acid water in funnel with pipette so that level of acid water is at junction of burette and funnel. *Caution:* Care must be taken that none of the gas sample escapes during this procedure (i.e. do not place pipette so that it sucks directly from capillary beneath funnel).

Adjust the mercury level so that the upper level of the gas sample is brought to the zero mark (i.e. the scratch mark between calibration and funnel). Read the level of the lower meniscus estimating to nearest ½ small division (1/200 cc.). This is Volume A.

7. Pipette about 1 cc. $N/10$ potassium hydroxide solution into the reservoir. *Note:* Use pipettes for only one solution (i.e. do not use the pipette with which the sulphuric acid was put into the reservoir for the potassium hydroxide).

8. Mix the potassium hydroxide with the gas sample by alternately lowering and raising the mercury level. The potassium hydroxide will run down the sides of the burette. Finally, lower the mercury so that the gas sample is brought down to the bulb. Measure the gas sample as above without removing excess potassium hydroxide. Mix again and remeasure to see that there has been no further absorption. If the two readings are identical, remove excess potassium hydroxide from funnel and make final reading of Volume (B).

Then $A - B$ = volume of carbon dioxide and $\frac{A - B}{A} \times 100$ = percentage of carbon dioxide.

9. Pipette anthraquinone solution into funnel and carry out the same procedure as in 8, for the absorption of oxygen. *Caution*: Do not leave anthraquinone solution unstoppered. Final volume is (C).

Then $B - C$ = volume of oxygen and $\frac{B - C}{A} \times 100$ = percentage of oxygen.

10. The final volume after absorption is (C). C = volume of nitrogen.

Then $\frac{C}{A} \times 100$ = percentage of nitrogen.

11. At the end of the analysis the apparatus is washed twice with 0.5% sulphuric acid. The pipette is filled with acid and is ready for the next analysis.

Precautions

1. Final measurements must always be done under the same conditions.

2. Do not warm the instrument with your hand. This will alter the volume of gas.

3. When readings are made, after mixing samples, wait for one minute to allow fluid within the burette to drain to the bottom.

4. In mixing, care must be taken that none of the gas sample escapes by bubbling through the reservoir.

THE LIPIDOLYTIC ENZYMES OF LARVAE OF *GALLERIA MELLONELLA*[1]

By Edith Mankiewicz

Abstract

The lipidolytic enzymes of *Galleria mellonella* and of pancreas are strongly contrasted by their activity on different substrates. The former is distinguished by its action on compounds of higher alcohols with lower fatty acids. More effective methods of extraction and preservation of the enzymes are described. The influence of emulsifying agents on the activity of the enzymes is demonstrated. The activity of *Galleria mellonella* enzyme on the wax of *Mycobacterium tuberculosis* is a particular feature.

More than three decades ago, Metalnikov discovered the exceptional ability of the digestive system and the serum of larvae of *Galleria mellonella* (L.G.M.) to digest wax and the waxy envelope of the tubercle bacillus. Although this observation suggests various possibilities, relatively few publications followed those of Metalnikov (9, 10, 11, 12, 13, 14).

In 1928, V. Pertzoff (15) extracted the powdered acetone-dried larvae with glycerol and sodium chloride solution, and he observed hydrolysis of olive oil and beeswax, and, at 45° C., a hydrolyzing action on the bacilli of tuberculosis. N. Fiessinger and A. Gajdos (6) tried unsuccessfully to purify the glycerol extract of the ground up L.G.M. by adsorption and elution, using the method of Willstaetter (17, 18). Their glycerol extract acted on tributyrin but did not act on olive oil. It was most active at room temperature and at pH 7.0 to 7.8. These authors pointed out one of the difficulties encountered when working with the glycerol extract of the L.G.M. to be the loss of more than 78% of its activity within the first 10 days after its preparation. C. Manunta and S. Carhoni (8) tested the fats extracted from the isolated intestines of the larvae to show that acidity, the saponification number, and the iodine value were all higher than those of beeswax, which confirms Metalnikov's observation of a digestion of alimentary wax in the intestine of the larvae. The nature of the lipases in the L.G.M. have not yet been well defined, but G. Clement and A. M. Frisch (4) found important quantities of lecithinase, which splits off the fatty acids of lecithin, and a cholesterase, which, at pH 7.2, converts cholesterol into its fatty acid esters.

The fact that the number of investigations of the lipidolytic enzymes of the L.G.M. is so restricted might readily be explained by the technical difficulties that are involved. To appreciate them it is necessary to consider briefly those that complicate the work on lipatic enzymes in general. Besides a quite remarkable confusion in the terminology concerning "esterases", "lipases", "hydrolysis", and "saponification*", many authors neglect to

[1] *Manuscript received March 5, 1949.*

* *Bull (3) recommended that the term saponification be limited to hydrolysis in which glycerol and soaps are obtained as the ultimate products, and the general name of hydrolysis be applied to all reactions in which fatty acids are split from glyceride with the addition of water.*

define the conditions of their experimental work. For instance, the controversy about the diminution of blood lipases during tuberculosis is due, at least partly, to the fact that different substrates have been used for investigations. According to our own experience in evolutive tuberculosis in guinea pigs, the various blood serum lipases do not diminish in the same proportion. Thus, in advanced cases of tuberculosis, the splitting of benzyl butyrate and ethyl butyrate show a marked decrease (loss of 28%), whereas the blood serum did not lose nearly as much enzymatic activity on olive oil (loss of 2%).

In reality, most of the lipolytic systems that have so far been studied represent a spectrum of activity, the composition of which varies with the conditions of experiment. To allow comparison and classification of the lipidolytic enzymes, we have to consider the following factors: method of preparation and degree of purification; activity on different substrates under various conditions of temperature and pH; resistance to antiseptics and inhibitors; and, as we shall see, the influence of emulsifying agents on activity and preservation of the preparation.

Some special difficulties are encountered in working with L.G.M., the first of which is to obtain a sufficient supply of larvae. An average of 20 to 30 adult larvae, ground and dried according to Willstaetter's method for pancreas powder, are needed to obtain 1 gm. of powder, or 16 cc. of glycerol extract. Through the kindness of Mr. S. M. Deschênes and Mr. Methot of the Department of Agriculture (Ottawa) infected beehives were obtained and the adult larvae collected.

After experimenting with different methods of extraction, including those of Willstaetter and Waldschmidt-Leitz, Fiessinger, Glick, and King, modified according to J. Desbordes (5), the extraction of 1 gm. of the powder by 16 cc. of pure glycerol at room temperature during three days seemed at first the most efficient method (Table I). However, later experiments proved the advantage of adding an emulsifying agent to the glycerol.

TABLE I

EXTRACTS OF THE ACETONE-DRIED POWDER OF L.G.M. IN UNITS PER 100 CC.

Substrate	Extractives		
	NaCl 0.85%	NaCl 10%	Glycerol
Ethyl-*n*-butyrate	100	60	307
Benzyl-*n*-butyrate	95	55	300
Tributyrin	210	155	540
Olive oil	90	70	290
Beeswax	55	20	310
Suspension of Tb bacilli	60	23	292

Note: Carboxel H.V. 1.5% as emulsifying agent during reaction. Initial pH 7.2. Titration after 24 hr. at 37° C.

Like Fiessinger, we failed in the adsorption and elution of the extract by aluminum hydrate.

In their *Studies of pancreatic function* (7), N. P. Goldstein and J. H. Roe discuss numerous methods for determining the activity of lipidolytic enzymes. The titration method using 0.1 *N* alcoholic potassium hydroxide, as proposed by these authors, was adopted as the most convenient, and sodium diethyl barbiturate was used as a buffer. Calcium acetate was added on the supposition that the fatty acids liberated in the hydrolysis form an insoluble calcium salt and that this removal of one of the end products prevents the reversal of the reaction. Using a standard amount, 1 cc. of the filtered enzyme preparation (equal to 1/16 gm. of powder), and allowing 24 hr. at 37° C. before titration, our only modification of the method proposed by Goldstein and Roe was the substitution of their emulsifying agent (bile glycerol) by gum of acacia or "Carboxel" when working with extracts of L.G.M. The results are expressed in arbitrary units determined by the number of cc. of 0.1 *N* potassium hydroxide to produce permanent pink to phenolphthalein end point minus the titration of the blank (containing heat-inactivated enzyme preparation) multiplied by 100: this figure gives the number of esterase or lipase units per 100 cc. of the preparation.

Comparison between the lipidolytic activities of glycerol extracts of beef pancreas and of L.G.M. are shown in Tables II and III.

TABLE II

COMPARISON OF HYDROLYTIC ACTIVITIES OF GLYCEROL EXTRACTS IN UNITS PER 100 CC.

Substrate	Beef pancreas		L.G.M.	
	Emulsifying agent during reaction			
	Glycerol–bile	Gum acacia 20%	Glycerol–bile	Gum acacia 20%
Ethyl-*n*-butyrate	310	150	120	307
Benzyl-*n*-butyrate	910	415	98	297
Tributyrin	1525	1350	150	514
Olive oil	550	515	150	266
Beeswax	0	40	115	300

Note: Initial pH 7.2. Titration after 24 hr. at 37° C.

It is evident from the results obtained that the lipidolytic activities of beef pancreas and L.G.M. do not cover the same substrates. Whereas the pancreas extract is most active on tributyrin, olive oil, and benzylbutyrate, the extract of L.G.M. has its highest activity on tributyrin and beeswax. The action of pancreas on tubercle bacilli is practically nil, and it has only little action on the alcohol–ether extract of the bacilli.

The specific differences in the compared activities of both extracts became more marked with the use of emulsifying agents other than bile glycerol. In

TABLE III

COMPARISON OF HYDROLYTIC ACTIVITIES OF GLYCEROL EXTRACTS IN UNITS PER 100 CC.

Substrate*	Beef pancreas		L.G.M.	
	Emulsifying agent during reaction			
	Glycerol–bile	Gum acacia 20%	Glycerol–bile	Gum acacia 20%
Suspension of tubercle bacilli in saline	10 (?)	0	85	278
Alcohol–ether extract of Tb bacilli ("Phosphatides – low melting wax–acetone soluble fats")	30	40	42	110
Chloroform–ether extract of Tb bacilli ("purified wax")	0	20	35	93
HCl – chloroform – ether extract, freed from HCl ("firmly bound lipids")	0	0	27	56

Note: Initial pH 7.2. Titration after 24 hr. at 37° C.

** A human strain of tubercle bacilli was grown on the medium of Proskauer and Beck, modified by Youmans and Karlson. Four- to six-weeks-old cultures were filtered, the bacilli were washed and thoroughly emulsified in saline. The fat fractions were prepared according to Anderson (1): the extracts were filtered and reduced. The values we indicate in these series of experiments have only comparative value: beef pancreas/LGM, since further purifications of the fractions might cause a shift in these "units".*

an attempt to intensify the lipidolytic action of the extracts, bile, bile salts, albumen, and gum of acacia were used.

On the kind suggestion of Prof. Labarre (Université de Montréal), we tried "Carboxel" (sodium carboxymethyl cellulose), which is a relatively pure product compared to gum of acacia.

The addition of bile, bile salts, and gum of acacia gave the following results:

(1). Bile solution in glycerol prepared according to Balls, Matlack, and Tucker (2) intensified the action of *pancreas extracts* on benzyl butyrate, and also on tributyrin, ethyl butyrate, and olive oil. Cholic acid was the most activating bile acid for pancreas lipases and esterases. (These findings are similar to those of Balls, Matlack, and Tucker (2), E. Rothlin and W. R. Schalch (16), and others.)

(2). The enzymatic activity of the *extracts of L.G.M.* was not favored by the addition of bile or bile salts, either in regard to their action on neutral fats or when the substrate was beeswax, emulsion of tubercle bacilli, or extracted tubercle bacilli fat fractions.

(3). Gum acacia, 20%, pH adjusted to 7.2, added to the *pancreas extracts* inhibited the cleavage of ethyl butyrate, benzyl butyrate, tributyrin, and slowed down the hydrolysis of olive oil, but allowed a faint action on beeswax, and on one group of lipoids extracted from tubercle bacilli.

TABLE IV

LOSS OF ACTIVITY OF VARIOUS EXTRACTS OF L.G.M. IN UNITS PER 100 CC.

Residual activity titrated after 24 hr. at 37° C. on various substrates. During the reaction, 1 cc. of each extract was emulsified in gum of acacia 20%. Initial pH 7.0 to 7.2

Days after preparation of extracts	Extractives					
	Glycerol	Glycerol + gum	Glycerol + carboxel H.V. 3%	Glycerol + carboxel L.V. 3%	Carboxel H.V. 3%	Carboxel L.V. 3%
Substrate: benzyl-n-butyrate						
4	297	505	725	794	753	706
14	270	358	655	599	580	519
40	150	210	627	510	541	510
Substrate: ethyl-n-butyrate						
4	307	358	1050	1089	1050	900
14	144	275	978	1005	1010	810
40	65	250	715	790	965	800
Substrate: tributyrin						
4	514	576	975	1146	1010	976
14	288	307	923	1033	949	860
40	285	302	920	890	620	730
Substrate: olive oil						
4	266	558	367	498	290	357
14	74	248	266	283	266	250
40	74	110	220	260	225	150
Substrate: emulsion of tubercle bacilli						
4	278	416	378	530	307	369
14	76	157	373	490	205	275
40	23	120	240	365	155	269

(4). Gum acacia added in the same proportion to the *extracts of L.G.M.* activated the cleavage of all the substrates, especially of benzyl butyrate, olive oil, beeswax, and the lipoids of the tubercle bacilli.

The following conclusions may be drawn from these observations:

(1). The lipidolytic enzyme system of L.G.M. contains lipases that are absent in beef pancreas, and that have a specific action on wax and waxy compounds.

(2). Biochemically, the specific action of L.G.M. on these substrates can be defined as an affinity for lipids formed by high alcohols with lower fatty acid chains.

In a more general way the findings suggest that the specificity of action of any lipase is determined by the alcohols of the lipid substrate rather than by the nature of the fatty acid chains.

(3). The difference in fat splitting enzymes of pancreas and L.G.M. is amplified by the activating or inhibiting properties of emulsifiers.

After thus establishing the special nature of the enzymes of L.G.M., "Carboxel" was found to be an even better emulsifying agent than those previously used. "Carboxel" is found on the market (Standard Chemical Company Ltd.) as a powder of high viscosity (HV) and low viscosity (LV). This chemical, used as emulsifier, activates the fat splitting process. Furthermore in 1.5% concentrations in 50% glycerol for the extraction of the L.G.M. powder, it retarded the deterioration of the enzyme in the extract. The best results so far obtained in regard to extraction and preservation of the lipases acting on high alcohol – low fatty acid compounds were when the L.G.M. powder was emulsified for three days at room temperature (pH 7.2) in Carboxel–glycerol, and the filtrates were kept in the Frigidaire. The glycerol–Carboxel extract of L.G.M. powder was more than twice as active on tributyrin as the glycerol–extract. While, 14 days after extraction, the lipidolytic activity of the glycerol–extract on tributyrin was diminished by nearly 50%, that of the glycerol–Carboxel extract was diminished by 10%. While in two weeks the glycerol extract lost around 75% of its activity on tubercle bacilli emulsion, the Carboxel–glycerol extract showed no appreciable loss of activity over the same period.

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THE RELATIONSHIP OF SERUM PHOSPHATASES TO SEX HORMONES[1]

By Jules Tuba, Donald B. Baker, and Max M. Cantor

Abstract

Castration produces a fall in serum inorganic phosphorus values in adult male rats but not in adult female rats. The alterations of phosphorus levels that occur following castration in male rats and after the injection of some hormones into normal and castrated rats of both sexes may be accounted for by corresponding alterations in metabolic requirements. Neither castration nor hormone injection produces any significant change in serum acid phosphatase activity in male or in female rats. A fall of about 30% to levels approaching values for normal female rats is found in serum alkaline phosphatase of castrated male rats in about eight weeks. There is no change in the enzyme values in castrated females. In those instances where injections of a sex hormone into castrated or normal rats produce alterations in alkaline serum phosphatase values such changes may be accounted for on the basis of altered food intake. The sole exception to this finding is the very marked decrease in the activity of the enzyme produced by progesterone after oestrogen in normal male rats.

Introduction

The investigations of the Gutmans (5, 7, 9), and of Huggins and Hodges (6) have demonstrated the importance of determining levels of serum "acid" and "alkaline" phosphatases both in the diagnosis of metastasizing prostatic carcinomas and following therapy with oestrogens or castration.

Observations on the effects of sex hormones on serum phosphatases have been carried out with experimental animals by Huggins and Hodges (6), and by Buchwald and Hudson (1, 2), but the results are scanty and conflicting. The investigations reported in this paper were carried out in a manner that controls a number of variable factors not usually considered and that can markedly affect the experimental data.

Experimental

Male and female castrated and intact adult albino rats (Wistar strain) were used. The castrated animals were left for an eight week postoperative period before being used for hormone injections.

During the development of the investigation it was found that in order to obtain normal mean values for serum alkaline phosphatase in intact rats it was necessary to consider the hour of day at which the animals were bled and the amount and type of food ingested. It was found that the levels of the alkaline enzyme dropped during the daylight hours and rose during the evening and night to reach a peak about 9 a.m. This diurnal variation is shown graphically in Fig. 1. It was also observed that if the animals were not fed their full daily requirements of approximately 27 gm. per day of Purina Fox Checkers their alkaline phosphatase levels were lower than those fed

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Contribution from the Department of Biochemistry, University of Alberta, Edmonton, Alta.

ad libitum. Subsequently Cantor, Wight, and Tuba (3) were able to show conclusively that it was the decrease in total daily fat ingestion, rather than the lowered calorie intake, that caused the lowering of the alkaline enzyme.

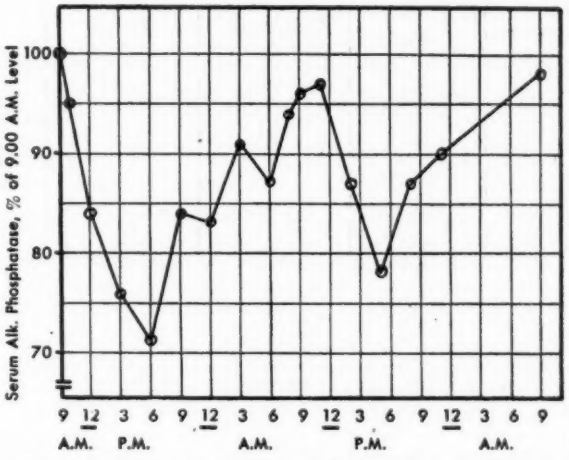

FIG. 1. *The daily variation in the serum alkaline phosphatase of adult male rats receiving adequate food.*

It was felt that the serum phosphatases of the female rat might be affected by the fluctuating hormone levels associated with various stages of the oestrous cycle. However, examination of a group of 30 adult females indicated that there was no significant alteration in levels of serum phosphorus or phosphatases of animals shown by vaginal smears to be in oestrus.

In view of the above findings, it was decided to feed the animals in excess of their requirements and to bleed them at 10 a.m. Using this regime, normal values for serum inorganic phosphorus, and for serum acid and alkaline phosphatases were established by means of the Shinowara, Jones, and Reinhart (8) micromethod as modified by Gould and Schwachman (4), and these are given in Tables I and II. It is important to note that the mean normal value of alkaline serum phosphatase for adult male rats is 113 units per 100 ml. of serum, and that this is markedly higher than the normal mean value for adult female rats of 70 units per 100 ml. serum.

In order to determine the effect of castration on serum inorganic phosphorus and phosphatases these were estimated once a week postoperatively for 8 to 12 weeks. Table III shows that in castrated male rats the serum alkaline phosphatase dropped about 30% from the normal level toward female values in approximately eight weeks. Beyond this time the alkaline phosphatase was found to fluctuate between 65 and 100 units, and the inorganic phosphorus decreased to lower than normal values. The acid enzyme showed no significant alterations.

TABLE I

THE MEAN, STANDARD ERROR OF THE MEAN, STANDARD DEVIATION, AND THE RANGE FOR SERUM INORGANIC PHOSPHORUS AND SERUM ACID AND ALKALINE PHOSPHATASES IN NORMAL MALE ADULT RATS

	Number of rats	Mean	Standard error of mean × 2	Standard deviation	Range
Serum inorganic phosphorus	141	8.0 mgm./100 ml.	± 0.2 mgm./100 ml.	± 1.2 mgm./100 ml.	5.4–12.4 mgm./100 ml.
Serum acid phosphatase	130	2.6 units/100 ml.	± 0.2 units/100 ml.	± 1.1 units/100 ml.	0.3–5.2 units/100 ml.
Serum alkaline phosphatase	151	113 units/100 ml.	± 5 units/100 ml.	± 28 units/100 ml.	35–189 units/100 ml.

TABLE II

THE MEAN, STANDARD ERROR OF THE MEAN, STANDARD DEVIATION, AND THE RANGE FOR SERUM INORGANIC PHOSPHORUS AND SERUM ACID AND ALKALINE PHOSPHATASES IN NORMAL FEMALE ADULT RATS

	Number of rats	Mean	Standard error of mean × 2	Standard deviation	Range
Serum inorganic phosphorus	120	7.2 mgm./100 ml.	± 0.3 mgm./100 ml.	± 1.5 mgm./100 ml.	4.7–15.5 mgm./100 ml.
Serum acid phosphatase	120	2.6 units/100 ml.	± 0.2 units/100 ml.	± 0.9 units/100 ml.	0.0–5.4 units/100 ml.
Serum alkaline phosphatase	122	70 units/100 ml.	± 4 units/100 ml.	± 21 units/100 ml.	34–128 units/100 ml.

TABLE III

THE EFFECT OF ORCHIDECTOMY ON SERUM PHOSPHATASES AND INORGANIC PHOSPHORUS. (MEAN OBTAINED FROM 10 RATS)

Number of weeks after operation	Serum inorganic phosphorus, mgm./100 ml.	Serum acid phosphatase, units/100 ml.	Serum alkaline phosphatase, units/100 ml.
Preoperative	8.0	2.3	106
1	8.2	3.5	103
2	8.5	2.4	96
3	8.4	2.6	96
4	8.1	4.2	95
5	8.2	2.8	80
6	8.1	3.7	72
7	8.0	1.0	69
8	7.9	2.5	63
10	7.2	3.2	85
12	5.8	2.0	96

In Table IV is shown the effect of öophorectomy on the serum phosphatases and phosphorus. The alterations here are essentially negative.

TABLE IV

THE EFFECT OF ÖOPHORECTOMY ON SERUM PHOSPHATASES AND INORGANIC PHOSPHORUS. (MEAN OBTAINED FROM 10 RATS)

Number of weeks after operation	Serum inorganic phosphorus, mgm./100 ml.	Serum acid phosphatase, units/100 ml.	Serum alkaline phosphatase, units/100 ml.
Preoperative	6.2	3.1	69
1	6.8	2.6	61
2	6.4	2.0	63
3	5.9	1.9	57
4	5.8	3.1	90
5	5.9	5.3	86
6	5.6	5.4	77
7	6.8	1.9	80
8	6.0	3.0	70

The sex hormones that were used to determine their effect on serum inorganic phosphorus and phosphatases of male and female rats were testosterone propionate, oestradiol dipropionate, diethylstilboestrol, progesterone, progesterone after oestradiol priming, and desoxycorticosterone acetate. Each hormone was tested on castrated animals and on intact animals. Groups of 10 rats were used for each test. The hormones, dissolved in oil, were injected intramuscularly in the dosages and at the time intervals shown in Tables V and VI. Desoxycorticosterone acetate, while not properly a sex hormone, was included because of its steroid nature.

Table V shows that significant changes are produced in the serum inorganic phosphorus level of male rats by some of the hormones. Testosterone injections caused a marked elevation of the typically low phosphorus levels of the castrates, but had no effect on normal males. Oestradiol, as well as diethylstilboestrol, produced a lowering of the phosphorus values in both normal and castrated male animals. Progesterone lowered the phosphorus in the serum of normal males and elevated the values in castrates. Injection of oestradiol on the second and fourth days of the experiment, followed by six daily injections of progesterone beginning the seventh day, produced a pronounced fall in normal male serum phosphorus similar to the effect of progesterone alone. Serum acid phosphatase results may be considered to be negative. Serum alkaline phosphatase values are significantly decreased by the female hormones in several instances. Oestradiol caused a decrease in castrates and normal animals. Diethylstilboestrol, progesterone, and progesterone after oestradiol produced lowered serum alkaline phosphatase activity in normal male rats.

Table VI indicates the changes occurring in the phosphorus levels in the serum of female rats. Testosterone and oestradiol increase the phosphorus

TABLE V

THE EFFECT OF SEX HORMONES ON THE SERUM PHOSPHATASES AND INORGANIC PHOSPHORUS OF ADULT MALE RATS. (EACH RESULT THE MEAN OF 10 RATS)

Sex hormone dosage	Number of days after initial injection	Number of injections	Serum inorganic phosphorus, mgm./100 ml.		Serum acid phosphatase, units/100 ml.		Serum alkaline phosphatase, units/100 ml.	
			Cast. males	Normal males	Cast. males	Normal males	Cast. males	Normal males
Testosterone propionate,	0	0	5.1	8.0	1.6	2.1	70	97
1 mgm./day/rat	15	14	10.8	7.8	0.5	4.3	62	75
	31	21	7.1	7.5	1.6	3.0	85	102
Oestradiol dipropionate,	0	0	6.3	8.5	2.4	3.1	98	114
0.1 mgm./day/rat	5	4	7.0	8.5	1.9	3.2	61	76
	9	6	5.9	6.2	1.2	3.8	40	61
Diethylstilboestrol,	0	0	6.5	8.8	1.8	2.4	58	122
0.1 mgm./day/rat	5	4	5.8	8.5	2.4	3.9	49	94
	9	6	5.6	6.8	3.3	4.0	68	82
Progesterone, 1 mgm./day/rat	0	0	5.7	10.1	2.4	3.1	76	115
	7	6	5.2	7.4	1.5	3.7	55	74
	14	12	8.9	6.5	1.9	4.5	66	66
Progesterone following oestradiol dipropionate,	0	0	5.4	10.3	3.1	3.1	90	102
0.1 mgm. oest./day/rat	7	2 oest.	4.5	8.4	1.9	2.3	42	39
1 mgm. prog./day/rat	14	6 prog.	5.5	6.6	2.0	3.6	59	31
Desoxycorticosterone acetate,	0	0	5.7	7.0	2.5	1.9	81	90
0.2 mgm./day/rat	7	6	6.5	6.2	1.4	2.4	70	82
Sesame oil, 0.2 ml./day/rat	0	0	5.9	8.8	2.1	2.4	83	116
	14	13	6.0	9.3	—	—	72	85

TABLE VI

THE EFFECT OF SEX HORMONES ON THE SERUM PHOSPHATASES AND INORGANIC PHOSPHORUS OF ADULT FEMALE RATS. (EACH RESULT THE MEAN OF 10 RATS)

Sex hormone dosage	Number of days after initial injection	Number of injections	Serum inorganic phosphorus, mgm./100 ml.		Serum acid phosphatase, units/100 ml.		Serum alkaline phosphatase, units/100 ml.	
			Cast. females	Normal females	Cast. females	Normal females	Cast. females	Normal females
Testosterone propionate,	0	0	4.9	7.9	2.3	2.5	78	83
1 mgm./day/rat	14	13	6.5	8.9	1.9	1.6	45	76
Oestradiol dipropionate,	0	0	5.8	7.0	3.5	2.4	62	71
0.1 mgm./day/rat	7	6	6.8	8.6	2.2	2.1	58	63
Diethylstilboestrol,	0	0	5.8	5.8	2.4	2.0	54	61
0.1 mgm./day/rat	7	6	6.3	7.3	2.6	1.8	26	43
Progesterone, 1 mgm./day/rat	0	0	6.5	7.2	5.0	3.5	76	67
	14	13	7.0	8.5	1.4	2.7	46	64
Desoxycorticosterone acetate,	0	0	6.9	7.1	3.1	2.7	85	86
0.2 mgm./day/rat	7	6	7.2	7.0	2.4	2.6	47	50

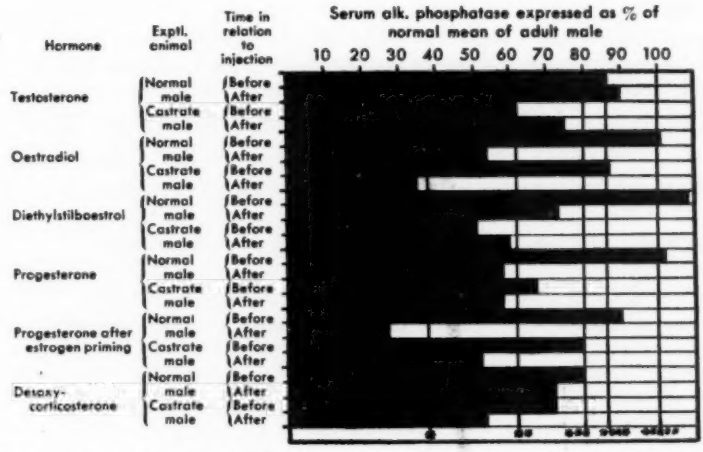

FIG. 2. *Serum alkaline phosphatase variations in the adult male rat resulting from the injection of sex hormones.*†

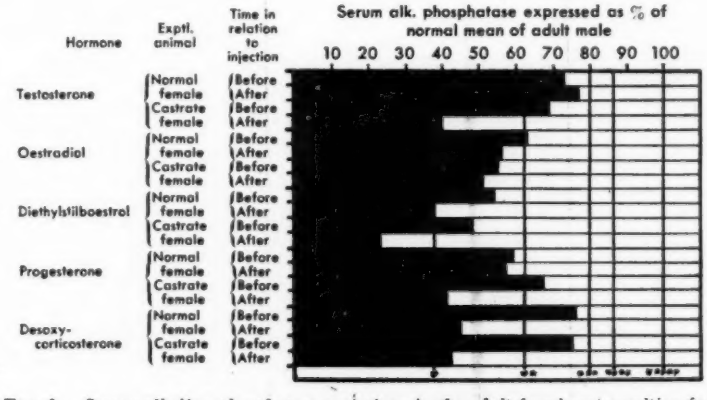

FIG. 3. *Serum alkaline phosphatase variations in the adult female rat resulting from the injection of sex hormones.*†

† NOTE: *Limiting values in Figs. 2 and 3 are calculated from the data of Tables I and II.*

* *Limit below which alterations from mean for normal female rats are significant.*

** *Mean for normal female rats.*

*** *Limit below which alterations from mean for normal male rats are significant.*

**** *Limit above which alterations from mean for normal female rats are significant.*

***** *Mean for normal male rats.*

levels in castrates and in normal rats. Diethylstilboestrol as also progesterone increases the values in the sera of normal female rats. Serum acid phosphatase changes are essentially negative. The only statistically significant fall in alkaline phosphatase is found in castrated females following injections of diethylstilboestrol.

Figs. 2 and 3 illustrate graphically the data given in Tables V and VI.

In order to determine whether the results obtained by hormone injections were due to the hormone per se or due to the amount of food ingested, a record was kept of the daily amounts of food eaten by the animals under various experimental conditions, and Table VII indicates these values.

TABLE VII

DAILY AVERAGE FOOD INTAKE OF ADULT RATS UNDER VARIOUS EXPERIMENTAL CONDITIONS REFERRED TO IN TABLES V AND VI

Condition of the experimental animal	Hormone injected	Amount of food ingested/rat/day in grams
Normal male		27
Normal female		15
Castrated male		Decreases from 27 gm. at time of operation to 15 gm. 8 weeks later. Marked fluctuations then occur
Castrated female		15
Normal males	Testosterone	23
	Oestradiol	10
	Diethylstilboestrol	10
	Progesterone	21
	Oestradiol and progesterone	10 during first week increasing to 20 in second
	Desoxycorticosterone	19
Castrated males	Testosterone	24
	Oestradiol	10
	Diethylstilboestrol	10
	Progesterone	20
	Oestradiol and progesterone	10 during first week increasing to 20 in second
	Desoxycorticosterone	11
Normal females	Testosterone	17
	Oestradiol	14
	Diethylstilboestrol	6
	Progesterone	15
	Desoxycorticosterone	15
Castrated females	Testosterone	11
	Oestradiol	8
	Diethylstilboestrol	4
	Progesterone	13
	Desoxycorticosterone	13

Attempts were then made to correlate the amount eaten by the animals with the levels of serum alkaline phosphatase, and the coefficient of correlation was calculated and found to be 0.70. This indicates that variations occurring in serum alkaline phosphatase due to castration or injection of sex hormones could be accounted for by an altered food intake. The one notable exception to this is the profound lowering of the alkaline enzyme level produced by progesterone after oestrogen priming in normal adult males.

Acknowledgments

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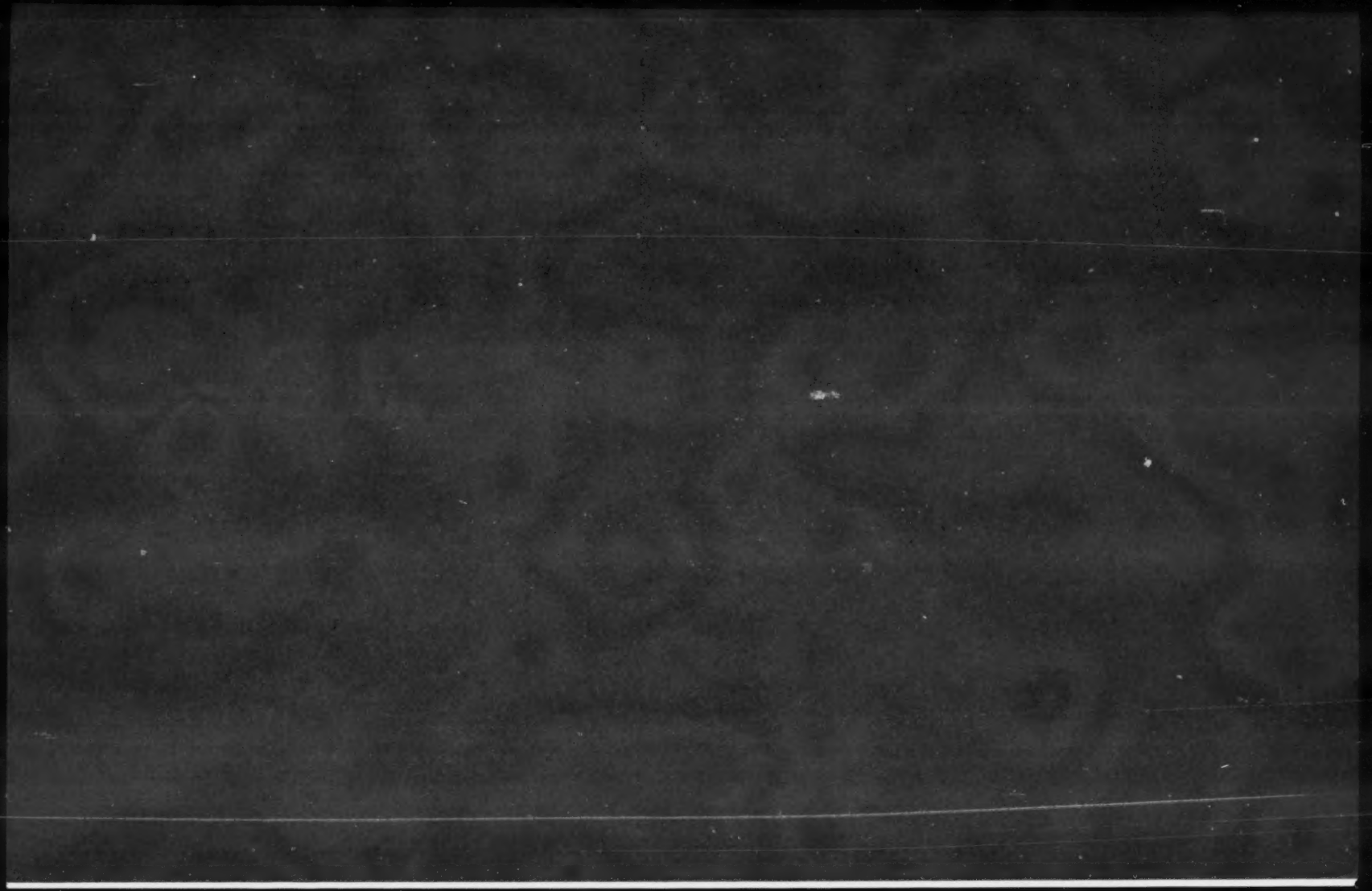
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